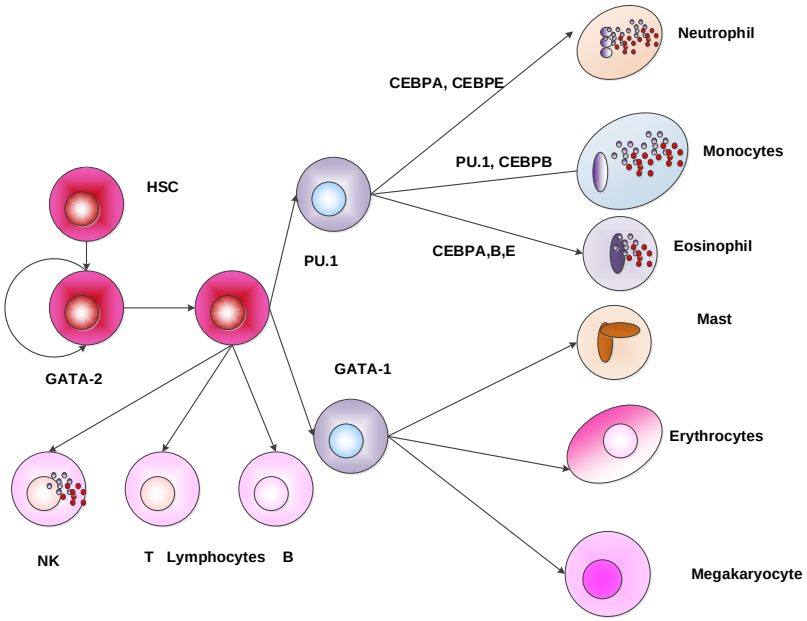

CANCER IMMUNOTHERAPY: A SYSTEMS APPROACH

DRAFT

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Notice

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Preface

The immune system has revealed itself slowly over the past century. In the 1950s, when I first became interested in Medicine, my earliest exposure was working in a Lab and seeing for the first time an explosion of lymphocytes in the blood smear of a young child. By that time I had read many slides, for my own education, but this one was amazing, red cells in a sea of lymphocytes. I looked to the Lab Director who asked me to examine this and wondered. She had a rather grave look on her face. The child was about ten and was out in the waiting room with her mother. I recalled the color of the sky, the late March day, the people huddled in their inexpensive wool coats, and the child. This would be a death sentence. It was leukemia. At that time, there was only a single outcome. And it would come quickly. That was my introduction to the immune system. Namely it was something to be feared, it could go out of control and destroy, destroy young children.

Going ahead some twenty-five years I began to follow Dr Steven Rosenberg at NCI. He was trying to take this potential flawed part of our human system and turn it into a weapon for the second flaw; cancer. Rosenberg had seen a patient with what was considered a terminal cancer and due to a massive infection, which was remedied and had somehow rid himself of the cancer. Cancer did not follow an inevitable one way path to death, it could be beneficial. Here was a dimension of the immune system which I had understood slightly from my studies, from patient data, but generally its inner workings were still a mystery. But through the use of new tools, methods to measure and means to change, the immune system was revealing itself.

Then came the use of immune therapy on melanoma, one of the most deadly of cancers. In the mid 1960s a melanoma was almost always a death sentence. But by the mid-2010s there were a multiplicity of therapeutics, and amongst them was an immunotherapeutic one. It appears that week after week new therapeutics are being delivered to treat cancers. Top amongst them are monoclonal anti-bodies. These derivatives of standard antibodies have been engineered to meet the requirements for a variety of applications.

New "tools" have become available whereby innovative approaches to using the immune system to treat cancers are being developed on a daily basis. Lives are being extended and new knowledge being obtained.

This work focuses on the immune system as just that; a system. Seventy-five years ago, radio engineers knew how to build receivers. They understood that when you heated an anode in a tube it gave off current. The current was electrons, but the details of the electrons were coincidental. None of the engineers really cared about Einstein and the photoelectric effect or the quantum mechanics of electrons. They knew you heated the anode and the cathode collected the electrons and current flowed. You could then put in a screen between the two and you could make small changes in the voltage on the screen and the net result was a large change in the current through the tube. It became a simple amplifier. Step by step it was modified to make a radio, radar, computers and the like. The engineer knew basic principles, but the basic principles were put to use to effect some integrated system. Engineers understood such things as; if the grid voltage

increases the cathode current decreased. Namely the typical paradigm was: if this goes up then that goes down, and when that goes down the other thing goes around. Mechanical engineers had been practicing this for a century by that time and Civil engineers since the Romans. The principle is simple; understand the elements in a system and understand how they effect and affect one another and they try to use these to solve a problem. That is what we are trying to do herein. We are not presenting science. We are not presenting medicine. We are presenting the elements and the interactions so that we can "engineer" a solution.

In "engineering" solutions we will always come up against something that just does not work that well. Namely our simple assumptions must be modified. Tubes work well at say a 50 KHz range. Tubes have problems at 500 MHz. Why? Well that requires science.

Thus, our intent herein is to present a system view of immunotherapy. We present elements and interactions and then examine applications to several cancers. This is clearly a work in progress. This is a continually updated Draft of a systems approach. I would expect that we can update this several times a year as progress is made. To any readers, your comments are requested. As with any "work in progress" there may be changes, deletions, additions and even gross re-evaluations of the systems.

My objective herein is to examine the immune system as a process which can be engineered to effect a solution. Suggestions, critiques, errors, and the like are always welcome since this is a work in progress. Also, my approach is to try where possible to rely upon primary sources, verbatim, assume a "fair use" wherein I then take the primary sources, integrate and comment on them, to create a more comprehensive tale. I feel that using direct primary sources is essential in a work of this sort because we want to continually rely upon what new understanding has been brought to the fore.

Terrence McGarty
January 2018

1 INTRODUCTION

The immune system is a complex and seemingly ever unfolding set of elements which for the most part keep the human species a viable one. There are times when it fails such as in many cancers. There are times when it over expresses itself as in auto-immune diseases. However and for the most part it works well. The challenge is to understand how it can work and using tools that are being developed and understood how it can be applied to manage human disease.

The objective of this document is NOT to present immunology, that has and is being done in a variety of highly competent and specialized documents. Furthermore it is also NOT to explore cancer immunology, again there are a wealth of documents on that front as well. The intent is to try to examine immunology and cancer as say an engineer would, culling the important principles, collecting the existing tools and then trying to apply them to specific problems specifically in cancers.

I have used the paradigm of the Trivium as a metaphor for this approach. Its emphasis comes from Galen and in turn many of the Fourteenth Century physicians who dealt with the reinterpretation of the work. The use is as a vehicle to interpret not a literality. As Niels Jerne stated in his Nobel lecture on 8 December, 1984:

Grammar is a science that is more than 2000 years old, whereas immunology has become a respectable part of biology only during the past hundred years. Though both sciences still face exasperating problems, this lecture attempts to establish an analogy between linguistics and immunology, between the descriptions of language and of the immune system. Let me first recall some of the essential elements of the immune system, with which I shall be concerned. In 1890, von Behring and Kitasato were the first to discover antibody molecules in the blood serum of immunized animals, and to demonstrate that these antibodies could neutralize diphtheria toxin and tetanus toxin. They also demonstrated the specificity of antibodies: tetanus antitoxin cannot neutralize diphtheria toxin, and vice versa. During the first 30 years, or more, after these discoveries, most immunologists believed that all cells of our body are capable of producing antibodies, and it took until the 1950's before it became clear, and until 1960 before it was demonstrated (13), that only the white blood cells named lymphocytes can produce antibodies. The total number of lymphocytes represent a little more than 1% of the body weight of an animal. …

At this point, I shall make a quotation from Noam Chomsky concerning linguistics: "The central fact to which any significant linguistic theory must address itself is this: a mature speaker can produce a new sentence of his language on the appropriate occasion, and other speakers can understand it immediately, though it is equally new to them … Grammar is a device that specifies the infinite set of well-formed sentences and assigns to each of these one or more structural descriptions. Perhaps we should call such a device a generative grammar … which should, ideally, contain a central syntactic component …, a phonological component and a semantic component." That is the end of my quotation. For the size of the set of possible sentences in a language, Chomsky uses the word "open-endedness", and I now think that "openended" is the best description also of the "completeness" of the antibody repertoire. As

for the components of a generative grammar that Chomsky mentions, we could with some imagination equate these with various features of protein structures. Every amino acid sequence is a polypeptide chain, but not every sequence will produce a well-folded stable protein molecule with acceptable shapes, hydrophobicity, electrostatics, etc. ….

Some grammatical rules would seem to be required. It is harder, however, to find an analogy to semantics: does the immune system distinguish between meaningful and meaningless antigens? Perhaps the distinction between "self' and "non-self' is a valid example. It would seem, at first sight, that the immune response to a sentence presented by an invading protein molecule is merely to select, from amongst its enormous preformed antibody repertoire, a suitable mirror image of part of this antigenic sentence. As you will know, Leonardo da Vinci wrote his private journal in the mirror image of ordinary handwriting. It is difficult, without considerable practice, to write and read mirror handscript.

This was one of the first times that a scientists seemed to connect grammar and the immune system. Yet from the time of Galen we know that he stressed the complete elements of the Trivium; grammar, logic and rhetoric. Why did Galen do so? Most likely for two reasons. First, as a Greek that may very well have been the basis of his initial education as well as that of his patients. Thus, it made for a modicum of communications. Second, there was an attempt to use deductive reasoning so that the conclusions were incontrovertible. The problem of course with the latter statement is that the four humors were truly without any scientific basis and the conclusions deductively drawn for treatment were built upon sand. In contrast current think is often inductive, which means we see what believe is a cause and then correlate it to an effect. We then tray to generalize the argument. Immunology is filled with these inductive judgements. Thus a review of the Trivium approach, subjecting it to inductions is useful.

Grammar: What are we saying and specifically with regard to the immune system. There are "things" and there are "actions" that occur in the system. There are subjects and there are predicates. Frankly it appears that each day we learn more about such elements. Thus the elements of this grammar, the grammar of immunology, are the individual parts, the cells, T and B, the receptors, ligands, antibodies, and signalling proteins. It is more than just identifying some "word" or molecule, it is the ability to understand what it does. Is it a noun or verb, an activator or repressor? Grammar than metaphorically is the identification of a thing, its function and where that function belongs and what it does.

Logic: This is the art of using Grammar properly and combining it in such a manner as to effect an acceptable conclusion. The syllogism is the classic example coming down to us from Aristotle. But it was Galen who emphasized how best to use the proper and correct Grammar, namely the observations of the correct things from a patient, then to effect a diagnosis and prognosis based upon the logical processing of these observations. Logic for the understanding of the immune system is the understanding on how the grammatical elements when combined go from cause to effect.

Rhetoric: Rhetoric is the utilization of Logic based upon Grammar in effecting a result, usually in an oral presentation. It is the process of getting the point across using the right words and presenting logically a correct argument. Rhetoric is the process of effecting the change. In a

similar manner the Rhetorical arm of our paradigm is the application of proper immunological elements in a logically consistent manner using the tools at hand in our metaphorical Rhetorical tool box to effect a change in a disease state. Rhetoric is the application of the Logic to the effecting of some cure.

Unlike the bench scientists who searches for a deeper understanding of the basic elements, or the scientists who seeks and develop better tools, and also the practitioner who applies the new insight with the new tools to effect a cure, our intent is to examine this as a totality, as a holistic whole, and in so doing see what we can understand as regards to this then as a day to day process. A process which enables cures for different diseases in different people. For all too often each cancer is individual. Thus, the metaphor of personalized medicine. Frankly all medicine is and has been personalized, we try here to accept that ab initio.

1.1 IMMUNE SYSTEM

First, we present a brief overview of the immune system and its operations. We will return for a bit more detail shortly. The question we first address is; how does the immune system kill off threats? Simple, but not so simple. There are two basic parts of the immune system; the innate and the adaptive. The innate is what kicks in first. Any threat and it attacks, often like a sledge hammer. Think of the common cold. The rhinovirus. It starts with the sensation in the nose, then one can almost feel as it spreads and then almost immediately the innate system kicks in with cytokines, who behavior is not really nice, with runny noses and sore throats. In contrast longer lasting threats get a chance to have the adaptive system kick in and create antibodies, such as having chicken pox or measles.

We demonstrate these below. Then for each we delineate the processes by which the immune system attacks the invader. The innate system may send out neutrophils and if it is a significant infection, such as a burst appendix, the immune system tries sending out "baby" neutrophils, called bands, and we get the "shift to the left" in our hematological analysis. Dead neutrophils become a pile of pus around the invader. The complement system may also kick in with its collection of cytokines. Phagocytosis may also become a process. Phagocytosis is the engulfment and degradation of microbes and other particulate matter by cells such as macrophages, dendritic cells, neutrophils, and even B lymphocytes (prior to their activation). These cells are part of the body’s “cleansing” mechanism. They not only de-fend the body by ingesting microbes, but also remove cellular debris and particulate matter that arise from normal physiologic functions. Phagocytosis involves cell-surface receptors associated with specialized regions of the plasma membrane called clathrin-coated pits. Dendritic cells use an additional mechanism to sample large amounts of soluble molecules, a process known as macropinocytosis. Last but in no way least is the attack of the NK, natural killer, cells. NK cells are fast killers of cells who do not belong. These are not to be confused with NK T cells which we will get to shortly.

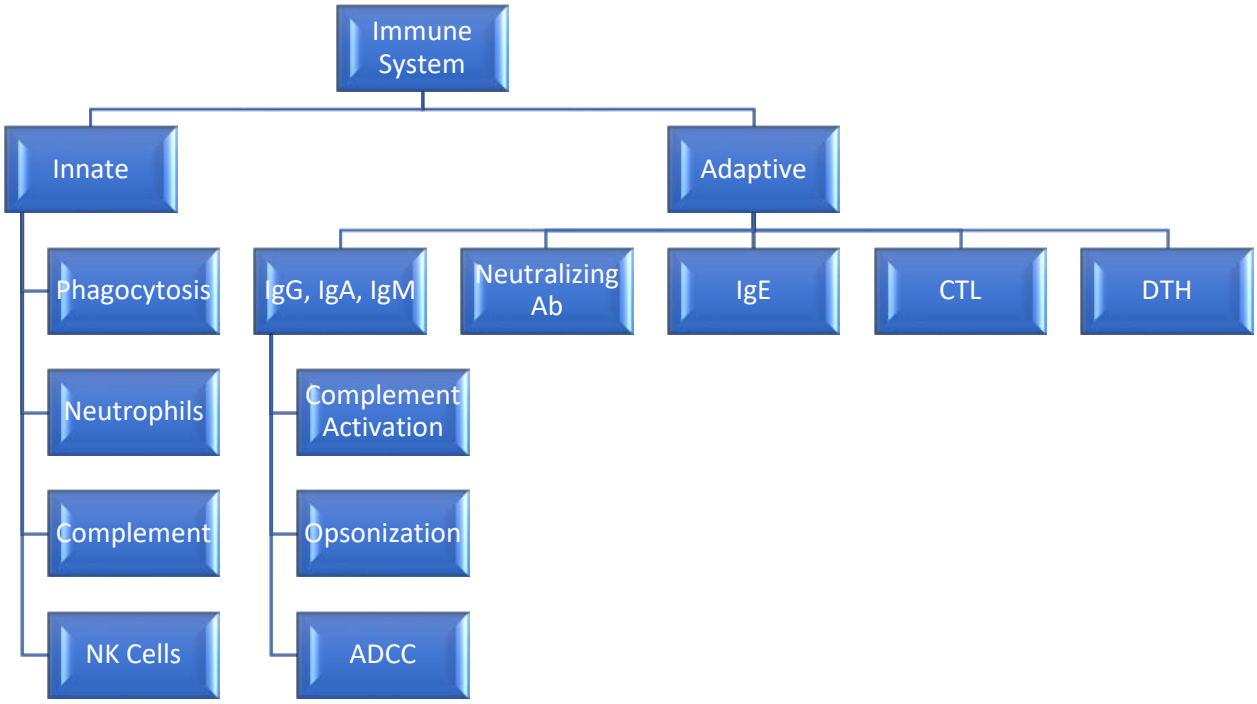
Then we have the adaptive system, the one where we have the development of anti-bodies, Ab. Below we show these elements. Using the Abs we can cover the intruder and mark them for elimination. Elimination may come about by a variety of means including use of the complement system.

For example, from Doan et al (p 143) we have:

Neutralization is the binding of antibodies to microbial epitopes or soluble molecules (e.g., toxins) in a manner that inhibits the ability of these microbes or molecules to bind to host cell surfaces. Binding to host cell surfaces is a necessary step for microbes and toxins to enter and damage host cells. Antibodies generated against the microbes (or toxins) often include some that block their interaction with the host cell surface, thus preventing the microbe (or toxin) from entering the cell. Neutralizing antibodies are usually of the IgG and IgA isotypes. It is the presence of neutralizing antibodies generated during the initial infections that provides the greatest protection against subsequent reinfection by the same organism. specific immunoglobulins that inhibit the infectivity of a virus or the toxicity of a molecule.

Furthermore, we have other ways in which Ab can function. Doan et al (p 146) state:

Antibody-dependent cell-mediated cytotoxicity or ADCC is the "tagging" of an invasive organism can attract phagocytic cells and other cytolytic cells. FcRs on NK cells and eosinophils. The bound cells may be bacteria, protozoa, or even some parasitic worms. As with phagocytic cells, these receptors al-low the cytolytic cells to bind invasive organisms "tagged" with IgG, IgE, or IgA antibodies, but rather than engulfment, they use cytolytic mechanisms to kill the "tagged" organisms. This process is termed antibody-dependent cell-mediated cytotoxicity (or as noted above, ADCC). The cytolytic mechanisms used by NK cells and eosinophils in ADCC are similar to some of those used by cytotoxic T cells to kill the intruder.



Thus, from a systematic perspective, we have the above list and categorization of means and methods to attack invaders, and in our case the invader is a cancer cell. We already know that approaches such as MAb, monoclonal Ab is an approach, NK and CTL design are another

approach. Substances such as interferons have been used since they tend to drive many of these immune attack processes.

Let us delineate these a bit more. Again, from Doan et al we have the following sets of Tables. The first Table below is for the innate system. Note that for a variety of pathogens we have one of the several innate attack mechanisms to work with. For cancer, we have experience using the NK cells which we shall discuss at length latter. The other three general categories have been tried in part. Various cytokines have been used with some limited results. It is difficult to target with the innate system. To some degree it is a "carpet bombing" approach.

Organisms	Representative.	Phagocytosis.	Neutrophils	Complement	NK Cells
Viruses (intracellular. cytoplasmic)	Influenza virus				
	Mumps virus				
	Morbillivirus (measles, rubeola)				
	Rhinovirus				
Bacteria (intracellular)	*Listeria monocytogenes*				
	Legionella spp.				
	Mycobacteria				
	Rickettsia				
Bacteria (extracellular)	*Staphylococcus* spp.				
	Streptococcus spp.				
	Neisseria spp.				
	Salmonella typhi				
Protozoa (intracellular)	*Plasmodium malariae*				
	L. donovani				
Protozoa (extracellular)	*Entamoeba histolytica*				
	Giardia lamblia				
Fungi (extracellular)	*Candida* spp.				
	Histoplasma				
	Cryptococcus				

The following Table is for the adaptive path. These involve some form of adapting to specific targets. They use Ab as markers for cells to be eliminated. The CTL, cytotoxic T cell, also called the killer T cell, and a list of other names is often a key vehicle.

	IgM, IgG, IgA						
Organisms	**Complement Activation**	**Opsonization**	**ADCC**	**Neutralizing Antibody**	**IgE**	**CTL**	**DTH**
Viruses							
Bacteria (intracellular)							
Bacteria (extracellular)							
Protozoa (intracellular)							
Protozoa (extracellular)							
Fungi							
Flatworms							
Roundworms							

1.2 TOOLS

A key to effecting therapies with the immune system is the concatenation of sets of tools to make it work. Tools are mechanisms or devices by which we can modify, implement, or otherwise assemble an immune response via the basic elements available. We present three significant ones but there are dozens of others being used or developed. Thus we present a construct with examples and not a compendium of all available. It is this collection of "tools" which become the key elements of using our knowledge of the immune system in toto.

We consider the three detailed in the Figure below:

CRISPR

- Using a CRISPR to target a specific section of DNA to be cut
- Using a protein like a Cas9 to "cut" the DNA at a desired point.
- Then to insert, delete, modify the DNA accordingly

Gene Drive

- A mechanism whereby a specific gene can be inserted into an organism and then replicate itself using a CRISP method and "drive" out any alternatives
- Generally this has been used in embryonic situations but applications to stem cells is possible in immune system

MAB

- The oldest approach whereby a specific antibody can be formed exvivo and then returned to the human host to excite the immune system to a specific target.

1.3 THERAPIES

Our third area of focus is to examine how knowing the basic elements, we can then by using a set of tools, effect a therapeutic treatment of a disease.

As Galluzzi et al note:

During the past decades, anticancer immunotherapy has evolved from a promising therapeutic option to a robust clinical reality. Many immunotherapeutic regimens are now approved by the US Food and Drug Administration and the European Medicines Agency for use in cancer patients, and many others are being investigated as standalone therapeutic interventions or combined with conventional treatments in clinical studies.

Immunotherapies may be subdivided into "passive" and "active" based on their ability to engage the host immune system against cancer. Since the anticancer activity of most passive immune therapeutics (including tumor-targeting monoclonal antibodies) also relies on the host immune system, this classification does not properly reflect the complexity of the drug-host-tumor interaction.

Alternatively, anticancer immune therapeutics can be classified according to their antigen specificity. While some immunotherapies specifically target one (or a few) defined tumor-associated antigen(s),others operate in a relatively non-specific manner and boost natural or therapy-elicited anticancer immune responses of unknown and often broad specificity. Here, we propose a critical, integrated classification of anticancer immunotherapies and discuss the clinical relevance of these approaches.

Galluzzi et al then present the following Table. On the left are a set of "tools" including those we focus on. On the right is their status.

Paradigm	***Licensed****
Tumor-targeting mAbs	YES
Adoptive cell transfer	NO
Oncolytic viruses	YES
Dendritic Cell-based interventions	YES
DNA-based vaccines	NO
Peptide-based vaccines	YES
Immunostimulatory cytokines	YES
Immunomodulatory mAbs	YES
Inhibitors of immunosuppressive metabolism	NO
PRR agonists	YES
CAR T Cells	Yes
ICD inducers	YES

2 THE IMMUNE SYSTEM

We provide a brief summary of the immune system focusing on specific elements related to immunotherapy. Our goal is not to present a full picture of what is known about the immune system. They are voluminous and is ever changing and growing. Our goal is to demonstrate the tools which may be available and some of which are used. The immune system is a powerful tool; however, it can be argued that much of its power is still being uncovered. The risk is that we may unleash a system that if uncontrolled can kill its current owner. For example, CAR-T cells, which we will discuss in the next sections can result in a cytokine storm, a massive number of cytokines released and flowing across all the body's organs reaping a maelstrom of damage.

Our approach to the immune system is a systems approach. Thus, it consists of the following general entities:

1. Elements: These are the basic set of elements which can be the cause and effect of the actions permitted.

2. Actions: What the results are of a combination of initiators and activators.

3. Initiators: These are facilitators of inter-Element actions.

4. Activators: These are the facilitators on the receiving side of actions initiated via Initiators.

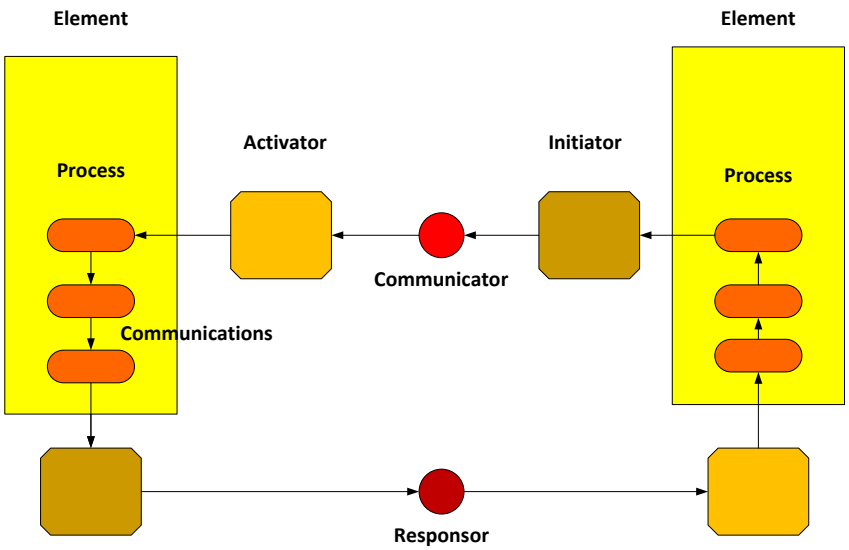
5. Communicator: The Communicator is the element which effects the interaction between Initiator and Activator.

6. Communications: Communications are the sub-elements which provide inter-element actions beyond just activators and initiators.

7. Processes: The Processes are the internal set of actions which connect impacts of Activators with Responses.

8. Responses: These are the resulting actions that result from the internal Processes.

Thus, we can consider this set of elements as demonstrate below.

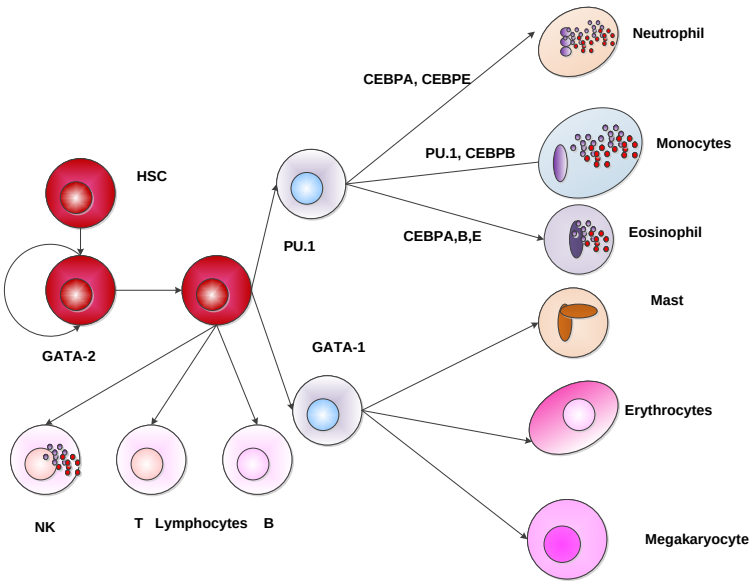


2.1 ELEMENTS

2.1.1 Blood Cells

Hematopoietic developments are fundamentally clonal in nature. The Figure below is a classic summary of the major players. We have added NK cells but have not provided details on differing T cells in the Figure. Basically, it all starts with a stem cell which is pluripotent for the cells in the line. Then depending on what influences the cell it develops into a myeloid or lymphoid line and ultimately into one of a plethora number of cells. The cells have varying lifetimes in the body with the red blood cells, which lack a nucleus, living about 90 days.

The following Figure is a graphic of classic development of blood cells, immune and otherwise. The development process is clonal from the bone marrow and the individual cell lines have maturation in various spots within the body. Generally, we look towards the lymphocytes as the core immune cells.



2.1.2 B and T Lymphocytes

The B and T lymphocytes are for the most part elements of the adaptive immune system. Namely they play the role of developing antibodies which in turn mark cells.

2.1.3 Phagocytosis

Phagocytosis is the engulfment and degradation of microbes and other particulate matter by cells such as;

1. macrophages,
2. dendritic cells,
3. neutrophils, and even
4. B lymphocytes (prior to their activation).

These cells are part of the body's "cleansing" mechanism. They not only de-fend the body by ingesting microbes, but also remove cellular debris and particulate matter that arise from normal physiologic functions. Phagocytosis involves cell-surface receptors associated with specialized regions of the plasma membrane called clathrin-coated pits. Dendritic cells use an additional mechanism to sample large amounts of soluble molecules, a process known as macropinocytosis. This process does not involve clathrin. Instead, plasma membrane "or" projections fold back on the membrane to engulf extracellular liquids in large intracellular vesicles.

1. Recognition and attachment of microbes by phagocytes: Phagocytosis is initiated when a phagocyte binds a cell or molecule that has penetrated the body's barriers. The binding occurs at various receptors on the phagocyte surface. These include PRRs (including TLRs) that recognize microbe-related molecules, complement receptors (CR) that recognize certain fragments of complement (especially C3b) that adhere to microbial surfaces, Fc receptors that recognize

immunoglobulins that have bound to microbial surfaces or other particles, scavenger receptors, and others.

2. Ingestion of microbes and other material: Following attachment to the cell membrane, a microorganism or foreign particle is engulfed by extensions of the cytoplasm and cell membrane called pseudo-podia and is drawn into the cell by internalization or endocytosis. In addition to phagocytosis, dendritic cells can extend plasma membrane projections and encircle large amounts of extra-cellular liquids to form cytoplasmic vesicles independent of cell sur-face attachment. Once internalized, the bacteria are trapped within phagocytic vacuoles (phagosomes) or cytoplasmic vesicles with-in the cytoplasm. The attachment and ingestion of microbe's trigger changes within the phagocyte. It increases in size, becomes more aggressive in seeking additional microbes to bind and ingest, and elevates production of certain molecules. Some of these molecules contribute to the destruction of the ingested microbes; others act as chemotactic agents and activators for other leukocytes.

3. Destruction of ingested microbes and other materials: Phagosomes, the membrane-bound organelles containing the ingested microbes/materials, fuse with lysosomes to form phagolysosomes. Lysosomes employ multiple mechanisms for killing and degrading ingested matter. These include; lysosomal acid hydrolases, including proteases, nucleases, and lipases; several oxygen radicals, including superoxide radicals, hypochlorite, hydrogen peroxide, and hydroxyl radicals that are highly toxic to microbes. The combined action of these molecules involves a period of heightened oxy-gen uptake known as the oxidative burst; nitrous oxide (NO) decreased pH; and other microcidal molecules.

4. Secretion of cytokines and chemokines: Once activated, phagocytes secrete cytokines and chemokines that attract and activate other cells involved in innate immune responses (see Table 5.2 and Tables 6.2 and 6.3). Cytokines or chemical messengers such as interleukin-1 (IL-1) and interleukin-6 (IL-6) induce the production of proteins that lead to elevation of body temperature. Other cytokines, such as tumor necrosis factor, increase the permeability of local vascular epithelia to increase its permeability and enhance the movement of cells and soluble molecules from the vasculature into the tissues. Still others, such as interleukin-8 (IL-8) and interleukin-12 (IL-12), attract and activate leukocytes such as neutrophils and NK cells. B.

2.1.4 Innate and Adaptive

At a high level, we see that the immune system has been divided into innate and adaptive. The innate system is classical and alternative, the latter being identified more recently. Both innate element result in the near immediate response of the immune system to pathogens. They use cytokines and other similar release elements to destroy the pathogen. The adaptive system is the longer-term approach wherein we see the use of antibodies generated through the antigen presentation to the B cells.

2.2 KILLER CELLS

There are three classes of "killer" cells, each somewhat distinct and providing different approaches to killing pathogens. They are: (i) Cytotoxic T Cells (or Killer T cells or CTL),

Natural Killer Cells (or NK cells) and (iii) Natural Killer T cells, NK-T cells. Each is different from the other and each has been used in a variety of ways in treating cancers[1].

2.2.1 CTL or Killer T Cells

These cells have MHC-I molecules and CD-8 surface proteins. They can be activated through the adaptive immune system. Activation is via IL-2 increase via T Cell helpers. CTLs can bind to a target cell a d they then can conjugate which allows for granule exocytosis which kills the target and then allows the CTL to progress to other targets. There are two pathways by which this attack can take; Fas pathway approach and the performin-ganzyme approach.

Pathogen recognition receptors, PRR, are the class of receptors which present in general terms proteins to the cell. Toll Like Receptors, TLR, function to transmit the presence of these noted ligands to the cell's nucleus where the DNA is activated to produce cytokines which then attack the cell.

As Steer et al note:

Although anti-cancer immunity involves both the innate and adaptive immune systems, it is generally held that CD8þ cytotoxic T lymphocytes (CTL) are the most potent anti-tumour effector cell. The T-cell immune response can be broken down into the following steps, all of which need to be fulfilled for effective anti-tumour CTL to be generated:

(1) tumour antigen(s) must be present, and

(2) these must be presented in a context which is seen as dangerous by the immune system;

(3) antigens must be acquired and presented by antigen presenting cells (APC) in the draining lymph node;

(4) specific T cells must then recognize and respond to tumour antigen by proliferating, exiting the lymph node, recirculating and entering the tumour as CTL and

(5) once within the tumour they need to overcome the local immunosuppressive environment before they can kill tumour cells.

In addition, memory cells may need to be generated to produce a sustained response. It is clear that a growing tumour has managed to escape this process. Failure of the anti-tumour immune response can occur at one or more of these steps. Targeting rate limiting steps with therapies designed to boost the immune response can improve anti-tumour immunity.

In addition to specifically targeted immune therapies, it is also now clear that many traditional cancer therapies can improve key aspects of anti-cancer immunity by inducing tumour cell death in a way that is immunostimulatory or by modulating tumour induced immunosuppression.

[1] See Kindt et al, Kuby Immunology, Freeman (New York) 2007; pp 353-368.

2.2.2 NK Cells

NK cells are not normal T cells and they deviate from the T cell line earlier. They amount for between 5% to 10% of the circulating lymphocytes. They work by producing cytokines and are generally considered a part of the innate immune system. They are considered part of the innate immune system.

NK cells have both activation and inhibition receptors. They act in such a manner as to becoming active or inactive by a balancing of activation, it is a thresholding effect. The NK cells have two types of receptors reflective of the NK cells requirement to balance activating and inhibiting receptor-ligand responses. The Killer Activation Receptors, KAR, are receptors which have the ability to recognize what are termed "stress" associated molecules, namely the ones which tell the NK cell that the cell should be considered to be attacked. In contrast, there is a Killer Inhibition receptor, KIR, which examines the MHC I molecule on the presenting cell to see if it is self. If the number of KAR activations exceed the KAR ones, then the NK cell attacks the cell. We shall discuss this later in some detail.

NK cells have the ability to sense activation and inhibition based upon what receptors are active on the surface. The inhibition is driven by seeing MHC I receptors on the surface of the interacting cell. The MHC I tells the NK that this is self. However, when a KAR receptor is activated then the perceived cells contains something that needs to be dealt with. But the NK cell has a multiplicity of inhibitors and activators and it is a process of some form of majority voting that results in the NK acting or not. We shall discuss this in detail later.

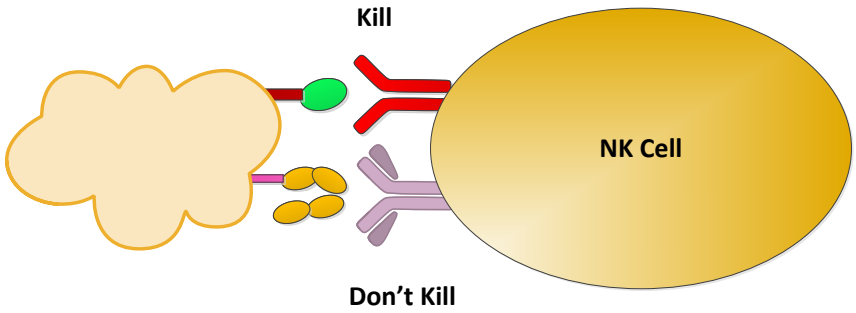
NK cells can be signaled by Interferons, TNF, IL-12 and IL-15. These have been used by researchers in attempts to activate the NK system.

The receptors are lechtin like or immunoglobulin like. The lechtin receptors bind proteins and not lechtins. The second receptors bind HLA-B and HLA-C.

There are inhibitory receptors which are immunoglobulin like such as ILT/LIR as well as KIR, called killer inhibitory receptors.

NK cells have activating and inhibiting ligands. Thus, an MHC-I represent a cell which is self and thus has an inhibitory reaction. A second receptor may reflect a viral infection and thus may activate. The actual activation is a balance between inhibition and activation. If the activation is strong enough then even though there may be an inhibitory self-recognition it may be overcome by the activating ligand. This may be a pathway for cancer management.

NK recognizes a marker on the surface and it decides to "kill" the cell. But it also recognizes the MHC I market as self and then does NOT Kill the cell. The MHC I acts as an inhibitor.



As Caligiuri notes:

Years ago, the histologic and functional definition of an NK cell was that of a large granular lymphocyte that could kill a target cell "naturally," that is, in a spontaneous fashion that did not require any priming and was not restricted by the target cell's expression of major histocompatibility complex (MHC) molecules. Experiments in mouse models of bone marrow graft rejection led to the proposal that NK cells would kill any target that lacked self–major histocompatibility complex (MHC) class I molecules (the "missing self" hypothesis).8 This extraordinary idea was developed before anyone knew what the NK cell was using to "see" its targets.

It is now clear that NK cells have a multitude of inhibitory and activating receptors that engage MHC class I molecules, MHC class I–like molecules, and molecules unrelated to MHC. Thus, NK cells are indeed restricted in what target cells they can engage by the expression of the target's MHC ligands, but in a very complex fashion that remains incompletely understood. Notably, orthologs of more recently discovered NK-cell receptor families cannot be found beyond mammals, suggesting that the composite modern day NK cell emerged well after T and B cells appeared to define the vertebrate adaptive immune system.

Furthermore, the complementary roles that NK and cytolytic T cells have in target recognition and host defense, and their similar mechanisms of cytolysis, suggest that these 2 cell types may have each evolved from a common ancestral cytolytic effector cell. Finally, a subset of human NK cells produce abundant cytokines with modest or no ability to lyse target cells. Thus, the older idea of an NK cell as an ancestral forerunner or as a cell defined by a simple function no longer applies. The traditional cell surface phenotype defining human NK cells within the lymphocyte gate on the flow cytometric analyzer shows an absence of CD3 (thereby excluding T cells) and expression of CD56, the 140-kDa isoform of neural cell adhesion molecule (NCAM) found on NK cells and a minority of T cells.

In contrast, murine NK cells do not express CD56, and … that NKp46, a member of the highly conserved natural cytotoxicity receptor (NCR) family of NK-activating receptors, best defines NK cells across species. Nevertheless, under close examination NKp46 can be found on a small subset of human cytolytic T lymphocytes.

Conversely, some CD56CD3 cells have low-density expression or may even lack expression of NKp46 (Figure 1); it will be interesting to determine the precise nature of these minority of cells. The search will no doubt continue for a sensitive and truly specific pan–NK-cell marker. Until this is found, the phenotypic definition of NK cells will continue to be determined by their expression of a unique combination of non– NK-restricted surface antigens.

He then goes on to describe what they do:

Thus far it has been fully appreciated that NK cells can secrete cytokines and chemokines that influence the host's immune response, and/or kill certain infected or transformed cells via perforin/granzyme or death receptor (egg, Fas, TRAIL)–related pathways.

Interferon gamma (IFN-) is considered the prototypic NK-cell cytokine, and its production by NK cells is known to shape the Th1 immune response, activate APCs to further up-regulate MHC class I expression, activate macrophage killing of obligate intracellular pathogens, and have antiproliferative effects on viral- and malignant-transformed cells. For many of these functions, it would make sense for NK cells to be in close proximity to APCs and T cells.

Indeed, the subset of NK cells that is the most potent producer of IFN- (i.e., CD56bright NK) is primarily located in the parafollicular T cell– and APC-rich region of SLT.21

As Pittari et al note:

The function of NK cells is governed by a set of germline- encoded activating or inhibitory receptors referred to as killer immunoglobulin-like receptors (KIRs). The extracellular domain determines which HLA class I molecule NK cells recognize, whereas the intracytoplasmic domain transmits either an activating or an inhibitory signal. KIRs are monomeric receptors with either 2 (KIR2D) or 3 (KIR3D) immunoglobulin-like domains, and are further subdivided into those with long (L) cytoplasmic tails (KIR2DL and KIR3DL) and short (S) cytoplasmic tails (KIR2DS and KIR3DS). Long-tail KIRs generate an inhibitory signal through the recruitment of the SH2-domain- containing tyrosine phosphatase 1 protein (SHP1) (8–11). Short- tail KIRs possess truncated portions that transduce activating signals via tyrosine phosphatase of DAP12 and other proteins.

As Vivier et al note:

NK cells were originally described as cytolytic effector lymphocytes, which, unlike cytotoxic T cells, can directly induce the death of tumor cells and virus-infected cells in the absence of specific immunization; hence their name.

Subsequently, NK cells have been recognized as major producers of cytokines such as interferon-g (IFN-g) in many physiological and pathological conditions.

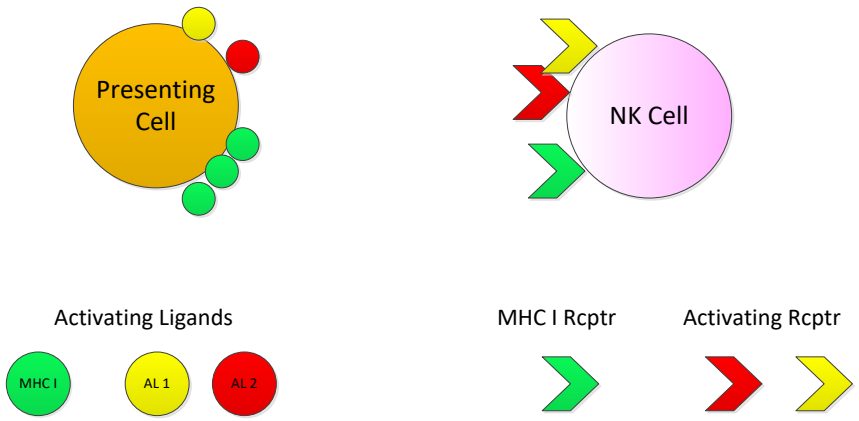
NK cells also produce an array of other cytokines, both proinflammatory and immunosuppressive, such as tumor necrosis factor–a (TNF-a) and interleukin (IL)–10, respectively, and growth factors such as GM-CSF (granulocyte macrophage colony-stimulating factor), G-CSF (granulocyte colony stimulating factor), and IL-3. NK cells also secrete many chemokines, including CCL2 (MCP-1), CCL3 (MIP1-a), CCL4 (MIP1-b), CCL5, XCL1 (lymphotactin), and CXCL8 (IL-8). Whereas the biological function of the growth factors secreted by NK cells remains to be clarified, their secretion of chemokines is key to their colocalization with other hematopoietic cells such as dendritic cells (DC) in areas of inflammation.

Furthermore, the production of IFN-g by NK cells helps to shape T cell responses in lymph nodes, possibly by a direct interaction between naïve T cells and NK cells migrating to secondary lymphoid compartments from inflamed peripheral tissues and by an indirect effect on DC (11) (Fig. 1).

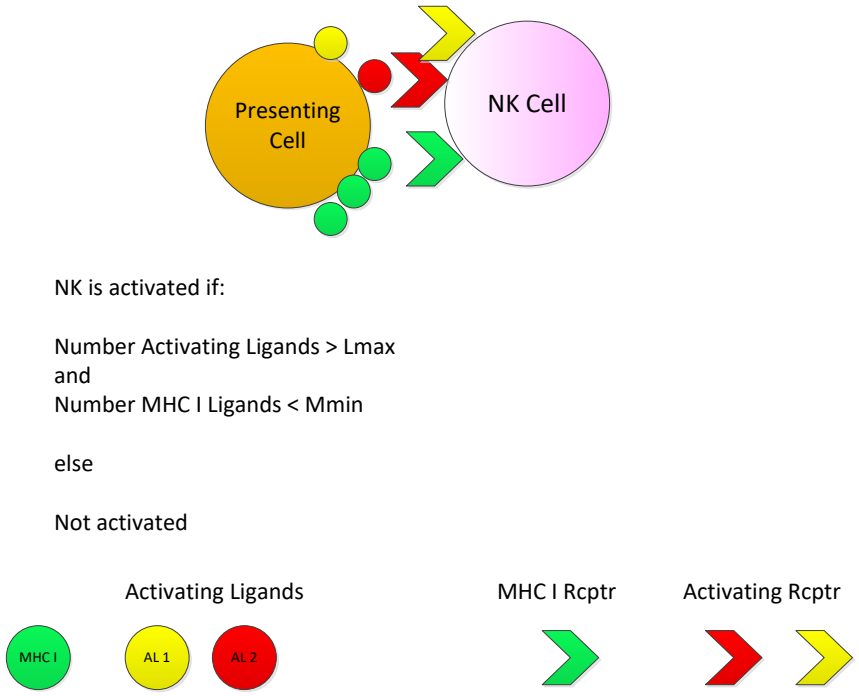
NK cell–mediated killing of target cells also impacts T cell responses, possibly by decreasing the antigenic load (12) and/or because target cell debris might promote antigen cross-presentation to CD8+ cytotoxic T cells (13) (Fig. 1). Although NK cells can positively (12, 13) or negatively (14) influence host T and B cell immunity, depending on the nature of the antigenic challenge, the emerging notion is that NK cells are not only cytolytic effector cells against microbeinfected cells or tumor cells. Rather, NK cell– mediated cytotoxicity and cytokine production impact DC, macrophages, and neutrophils (10) and endow NK cells with regulatory function affecting subsequent antigen-specific T and B cell responses.

Conversely, the "natural" effector function of NK cells has been revisited. NK cells require priming by various factors, such as IL- 15 presented by DC (15) or macrophages (16), IL-12 (17) or IL-18 (18), to achieve their full effector potential, highlighting the intimate regulatory interactions between NK cells and other components of the immune response. Thus, NK cells, like T and B cells, participate in the immunity in many different ways and undergo a process of functional maturation to fulfill these functions.

Now Vivier et al have described the rather interesting manner in which NK calls can be activated or inhibited. Simply, it is a bit of majority voting by ligands and receptors. We demonstrate this below. Activating ligands can attach to receptors as equally as inactivating.



Then below we demonstrate a somewhat simple majority voting scheme whereby the combination, subject to some putative weighting, can effect either activation or inactivation.



As Vivier et al note:

NK cells are equipped with an array of receptors that can either stimulate NK cell reactivity (activating receptors) or dampen NK cell reactivity (inhibitory receptors) (19, 20). Activating receptors include receptors that interact with soluble ligands such as cytokines and receptors that interact with cell surface molecules (Fig. 2).

Cytokine receptors that are coupled to the common gamma chain (gc), such as IL-15R, IL-2R, and IL-21R, are involved in NK cell development and effector function. In particular, IL-15 is required for the maturation and survival of NK cells, consistent with the absence of circulating NK cells in SCIDX1 patients and in mice lacking IL-15 or IL-15R components (21). Cytokine

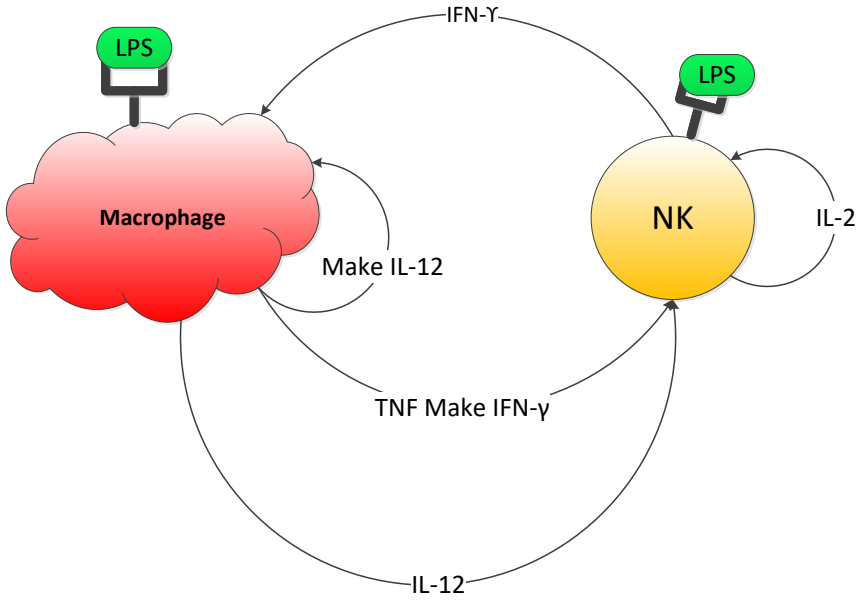
receptors that are linked to the adapter protein MyD88 are also important for NK cell maturation, namely IL-1R in humans (22) and IL-18R in the mouse (18).

NK cells exert their biological functions by various means. NK cells can kill a variety of target cells, including virus-infected cells and tumors, in the absence of antibody. In the case of viruses, the mouse Ly49H activating receptor recognizes a cytomegalovirus-encoded ligand (m157) (23, 24), and NKp46 has been reported to interact with hemagglutinins derived from influenza and parainfluenza viruses (25).

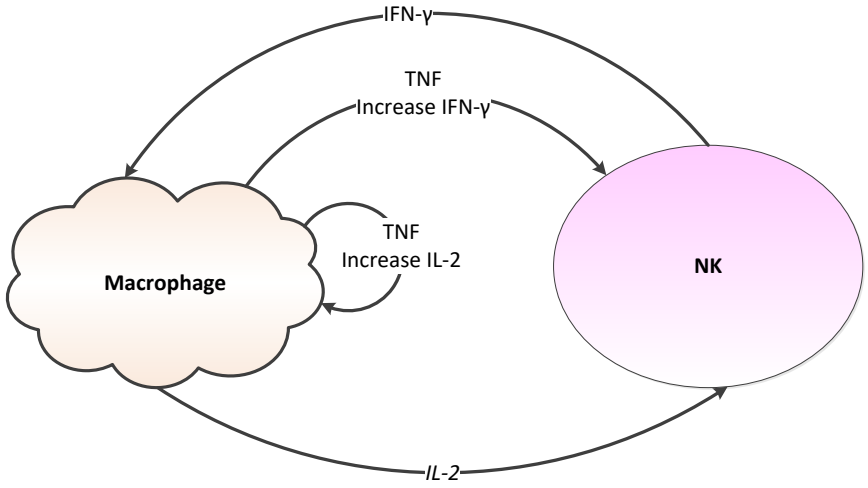
NKcells are also able to detect antibody-coated cells through the FcgRIIIA (CD16) cell surface receptor and to exert antibody-dependent cell cytotoxicity (ADCC) and cytokine production. CD16 is coupled to the CD3z and FcRg signal transduction polypeptides bearing intracytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs).

The natural cytotoxicity receptors (NKp46/NCR1, NKp44/NCR2, and NKp30/ NCR3) are also potent activation receptors linked to the ITAMbearing CD3z, FcRg, or DAP12 molecules (26). In mice, the NK1.1 (Nkrp1c) molecule on CD3– cells have been a useful marker for NK cells, but its expression is confined to only certain strains of mice. NKp46 appears to be the most specific NK cell marker across mammalian species, although discrete subsets of T cells also express it

Now shown below we depict the result of this activation process. There is a flow of Interferons further activating the NK and with the macrophage introduction of a pathogen identifier, in this case a lipo-poly saccharide, LPS, we see the NK then activated and beginning its response.



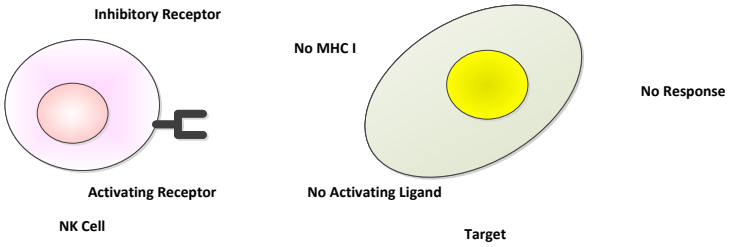
The figure below is another depiction of this process.

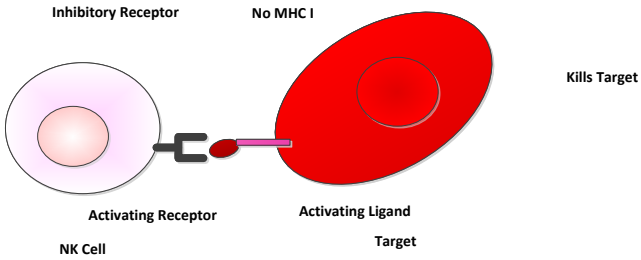


Vivier et al summarizes the various NK cell receptors by function.

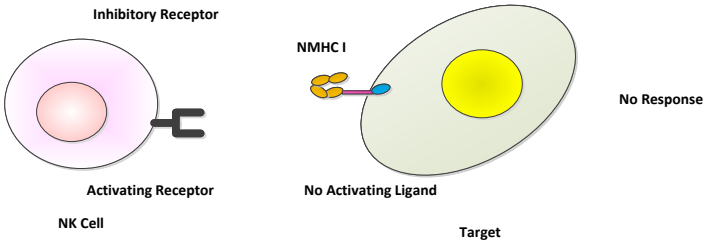
Function/ Receptor	Activating Receptors	Inhibitory	Cytokine Receptors	Adhesion Receptors	Activating Adaptors
	NKp46	h KIR-L	IL-1R	CD2	CD3ζ, FcRγ
	CD16	h LILRB1	IL-2R	DNAM-1	CD3ζ, FcRγ
	h NKp30	CD94/NKG2A	IL-12R	β1 integrins	CD3ζ, FcRγ
	h NKp44	m Inh. Ly49	IL-15R	β2 integrins	DAP12
	h NKp80	m NKR-P1B	IL-18R		FcRγ
	m NKR-P1C	m NKR-P1D	IL-21R		DAP10
	NKG2D	KLRG-1	IFNAR		DAP12
	m NKG2D-S	TIGIT			DAP12
	h KIR-S	CEACAM-1			DAP12, DAP10
	m Act. Ly49	LAIR-1			DAP12
	CD94/NKG2C				SAP, EAT2
	CRACC				SAP
	Ly9				SAP, EAT2
	CD84				SAP
	NTBA				SAP, EAT2,
	2B4				ERT

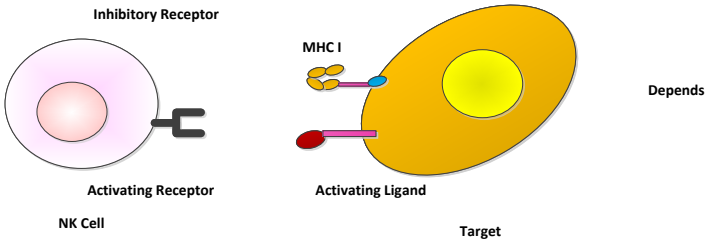
This is clearly a complex set of receptors which serve a multiplicity of functions. Vivier et al also discuss the question of NK being adaptive as well as innate. NK cells are quite powerful and have become cells of interest in a variety of cancer immunotherapeutic applications as we shall show latter.





The second set of elements and examples is shown below.





Thus, the set of states and their activations is depicted in the Table below.

	Target Activating On	Target Activating OFF
Target Inhibitory On	Depends on Balance	NK Attacks
Target Inhibiting Off	No Response	No Response

2.2.3 NK T Cells

The NK T cell is neither a CTL nor an NK cell. It is a third variety somewhat in between. CTL are adaptive and NK are innate. The T cell receptor on NKT cells does not recognize MHC molecules and it has markers similar to both NK and CTL.

As Ibarrondo et al note:

Invariant natural killer T cells (Type I NKT cells or iNKT) are a subset of T cells that express a restricted repertoire of T-cell receptors (TCR); in humans, the iNKT TCR alpha chain presents a Va24-JaQ rearrangement that preferentially pairs with a semiinvariant Vb11 b-chain. The iNKT TCR recognizes glycolipid antigens presented by CD1d, a major histocompatibility complexlike molecule present on the surface of antigen-presenting cells, and that is highly expressed by myeloid dendritic cells (mDCs). iNKT cells are actively recruited to infection sites, where they respond to cytokines and interact with CD1d + mDC [5]. In response to stimuli, iNKT cells can release large amounts of regulatory cytokines and are believed to play a pivotal role in the determination of innate and adaptive immune system responses.

iNKT cells can be subdivided into three subsets: CD4 +, CD8 + and CD42/CD82 double negative (DN). The CD4 + subset has a Th0 profile, being able to produce Th2 and Th1 cytokines such as interleukin 4 (IL-4) and interferon gamma (IFN-c). DN iNKT cells produce large amounts of Th1 cytokines such as INF-c and tumor necrosis factor alpha (TNF-a), up-regulate perforin, and release low levels of Th2 cytokines in response to stimuli [7]. Finally, CD8 + iNKT cells constitute a Th1-only subse. The balance of CD4 + versus DN and/or iNKT CD8 + iNKT cells is thought to be critical for proper modulation of immune responses to control inflammatory processes, auto-immunity, and immune surveillance of cancer. The pivotal role of iNKT cells in the regulation of the immune response makes them an attractive target for immunotherapy: the frequency and functionality of iNKT cells is frequently altered in patients with malignancies, autoimmune disorders, and viral infections. Blood iNKT cell frequencies fall in melanoma

As Stetson et al note:

Natural killer (NK) and NK T cells are tissue lymphocytes that secrete cytokines rapidly upon stimulation. Here, we show that these cells maintain distinct patterns of constitutive cytokine mRNAs. Unlike conventional T cells, NK T cells activate interleukin (IL)-4 and interferon (IFN)-

transcription during thymic development and populate the periphery with both cytokine loci previously modified by histone acetylation. Similarly, NK cells transcribe and modify the IFN- gene, but not IL-4, during developmental maturation in the bone marrow. Lineage specific patterns of cytokine transcripts predate infection and suggest evolutionary selection for invariant but distinct types of effector responses among the earliest responding lymphocytes. NK cells are required for effective host defense against herpes viruses in mice and humans.

Although the precise evolutionary niche subserved by NK T cells is not completely clear, the capacity of NK T cells to activate rapid cytokine expression has been exploited to manipulate the outcomes of autoimmunity and cancer. Aside from their expression of common NK-associated surface antigens, such as NK1.1, NK T and NK cells share developmental requirements. Deficiencies in certain cytokines, such as IL-15 or lymphotoxin, or transcription factors such as Ets-1 or Irf-1, lead to loss of both cell lineages. Recent studies suggest their capacity to express cytokines rapidly may also be developmentally acquired.

Although other studies elegantly demonstrate how these cells become activated, the mechanisms underlying their rapid cytokine production or their distinct cytokine patterns, IFN- in the case of NK cells and both IL-4 and IFN- in the case of NK T cells, remain unknown. Elucidation of such mechanisms may have important implications for understanding polarized cytokine production by T cells in adaptive immune responses.

We demonstrate that NK T cells and NK cells, distinguished by their ability to mobilize effector cytokines rapidly after immunization or infection, reside in the periphery spontaneously poised with constitutive cytokine transcripts.

Modification of the respective cytokine loci in a manner promoting access by transcription factors correlates with the presence of cytokine mRNAs. Unlike conventional T cells, NK T and NK cells activate transcription of cytokine genes during early development in the thymus and bone marrow, respectively. In the case of IL-4 for NK T cells, neither the percentage of IL-4

As Li et al note[2]:

NKT cells are highly heterogeneous effector cells with immune and regulatory functions. They possess the characteristics of both NK cells (CD56+) and T lymphocytes (CD3+). NKT cells are very rich in the mouse liver and account for 50% of intrahepatic lymphocytes, mainly located at the hepatic sinusoids. They play an important role in the prevention of tumor metastasis and the removal of viruses. It was shown, by a mouse hepatitis model induced by Con A or LPS plus IL-12, that NKT cells could induce liver damage, and the activated NKT cells disappeared from liver. The activation of NKT cells is often accompanied by the activation of NK cells, which then release cytokines that prevent viral replication. Therefore, it is thought that NK cells might be effector cells for NKT cells.

2.2.4 Comparison

[2] Li et al, Dynamic changes of cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, and natural killer T (NKT) cells in patients with acute hepatitis B infection, Virology Journal 2011, 8:199

The following Table compares the different cell types as discussed above.

	NK cell	Cytotoxic T cell	NK T Cell
Receptor type	NK receptor (numerous activating or inhibitory)	T cell receptor	
Ligand type	Class I MHC, MICA/B, immune complexes, etc.	Peptide-MHC class I complex	
Absence of class I MHC results in...	Immediate cytotoxicity ('missing self')	Lack of recognition	
Presence of class I MHC results in.	Inhibitory signal to NK cell	TCR engagement	

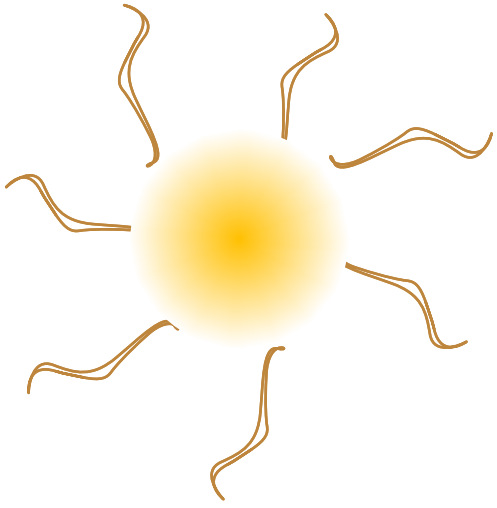
2.3 Antigen Presenting Cells (APC)

We now summarize the cells which present antigens, Ag.

2.3.1 Dendritic Cells

The dendritic cells are cells in the immune system which have branches, thus the dendron, and flow throughout the body collecting information on foreign invaders and presenting these to the immune cells. They present the antigens to the effector immune cells and start the immune process off against the invader. One of the first immunological approaches using the dendritic cells, DC, is its use on castrate resistant prostate cancer, and sipuleucel. We shall proceed to examine this approach in detail later (see Pendergast and Jaffee, Chpt 18).

The dendritic cells are named for the tree like or branched structure they look like as depicted below. (δενδρον)



As Lubong and Bhardwaj note:

Dendritic cells (DCs) are often called nature's adjuvants because of the way in which they help to initiate an immune response. Found throughout the body, the cells acquire and process antigens (the molecules recognized and bound by antibodies) from pathogens and tumors.

They then migrate to lymph nodes and activate T cells, which in turn induce protective immune responses. These properties have driven attempts to develop vaccines containing DCs loaded with tumour antigens, with the aim of inducing antitumor immune responses in patients with cancer.

But this strategy has fallen short of expectations… simply improving DC migration to lymph nodes dramatically enhances antitumor responses in humans and mice, pointing to a way to optimize the use of DC vaccines. There is a general consensus that DC vaccines can safely induce long-lasting antitumor immune responses. These vaccinations have produced encouraging, if modest, clinical results in some patients with advanced cancers. For instance, the vaccine sipuleucel-T (the only cell-based cancer vaccine approved for use in the United States) increases median survival times by four months in patients with prostate cancer.

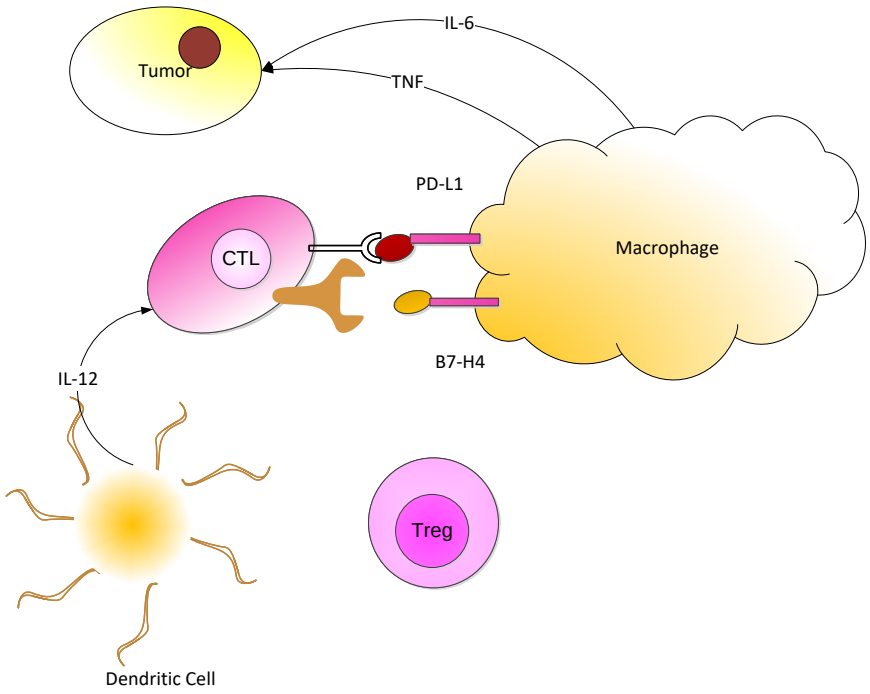
But several factors might be limiting the efficacy of DC vaccines: the source and type of DCs used; the site and frequency of injection; and the ability of DCs to migrate to lymph nodes. Moreover, the injected DCs may not themselves directly instigate an immune response, but instead might act indirectly through DCs already present in the lymph node. Less than 5% of cells in a DC vaccine reach the lymph nodes.

In mice, DC migration can be improved either by injecting activated DCs or by pre-conditioning the vaccination site in the skin with the inflammatory molecule TNF-α. Mitchell and colleagues therefore investigated whether pre-conditioning the DC vaccine site to generate local inflammatory responses might enhance DC migration in humans. To do this, they used a tetanus/diphtheria (Td) toxoid vaccine.

Most people have been exposed to this toxoid during childhood vaccinations, and re-exposure activates a subset of T cells called memory CD4+ T cells that recognize only the Td antigen and mount a strong and rapid inflammatory immune response in its presence.

2.3.2 Macrophages

Macrophages are out collecting Ag as well. A typical example of a macrophage action is depicted in the Figure below where we see it presenting to a cytotoxic T cell, CTL, and also producing tumor necrosis factor as a result of that activation.



From Ruffell and Coussens we have:

Macrophages are represented in all tissues by functionally and phenotypically distinct resident populations that are critical for development and homeostasis. Under nonpathological conditions, most resident macrophage populations derive from embryonic progenitors and are maintained through local proliferation. Exceptions to this include intestinal, dermal, and alveolar macrophages at barrier sites and macrophages in the adult heart that are replaced by circulating bone marrow-derived Ly6C+ inflammatory monocytes over a timescale of several weeks.

Under pathological conditions, there is evidence for both local proliferation and recruitment, with differences observed by tissue location and type of inflammatory insult….

For many solid tumor types, high densities of cells expressing macrophage-associated markers have generally been found to be associated with a poor clinical outcome. There are conflicting data for lung, stomach, prostate, and bone, where both positive and negative outcome associations have been reported, possibly related to the type/stage of cancer evaluated, or to the type of analysis performed. Some discrepancy may also reflect the use of different macrophage markers.

CD68, a glycoprotein predominantly resident in intracellular granules, represents a fairly specific marker for murine macrophages and, in combination with F4/80, identifies a majority of tumor-associated macrophages. In humans, however, CD68 expression is widespread and includes granulocytes, dendritic cells, fibroblasts, endothelial cells, and some lymphoid subsets…

2.3.3 Neutrophils and Mast

Neutrophils and Mast cells are also part of this process but we will not detail them at this time.

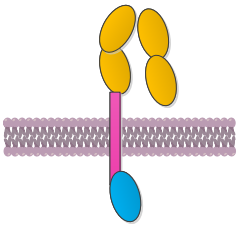
2.1 ANTIBODIES

Antibodies are a set of molecules produced by a B cell in response to antigens. They are the principal element of the adaptive immune system. When activated, they are produced in numbers and proceed to attack cells which are perceived a threat and establish multiple paths to the invaders destruction.

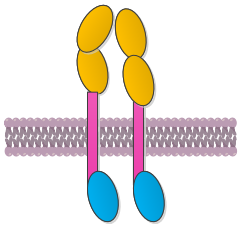
Antibodies can be made for specific antigens and antibodies have flexibility in their own lifetimes. We briefly discuss them here and leave detailed analysis to the literature. However, we use this as a way to introduce monoclonal antibodies, specifically because of their use in many immunotherapeutic applications.

2.1.1 MHC

The MHC, major histocompatibility complexes are a set of surface molecules on cells which are used to "present" antigens. There are fundamentally two types; MHC-I and MHC-II. MHC I appear on all cells whereas the MHC-II are more limited.



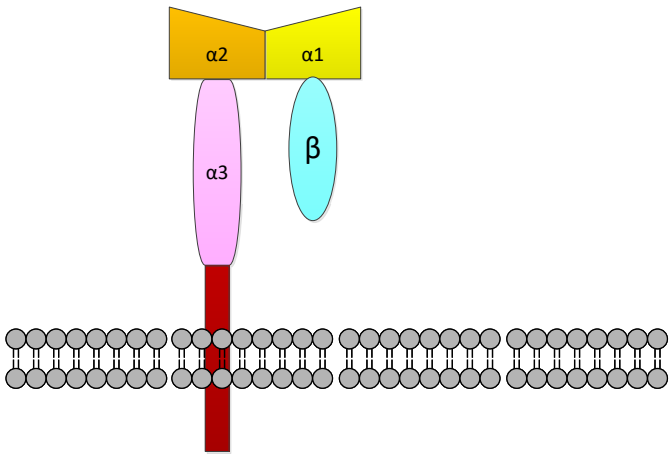
MHC I
All Cells
When something
NOT OK IN Cell



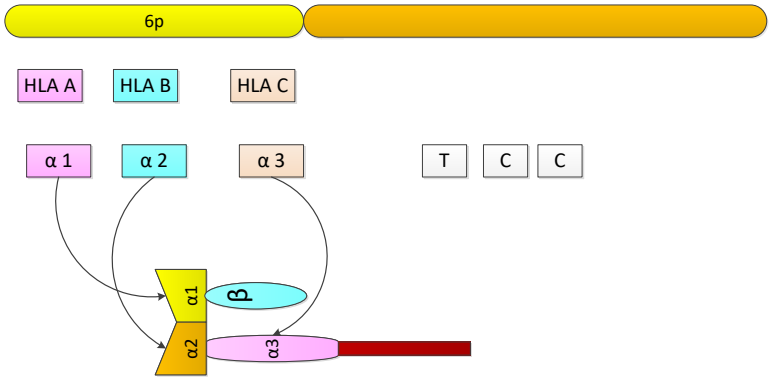
MHC II
APC Cells Only
Tells something
NOT OK Outside
Cell

Note the difference in cell interface. MHC II appears as a dimerized molecule with two intracellular action sites.

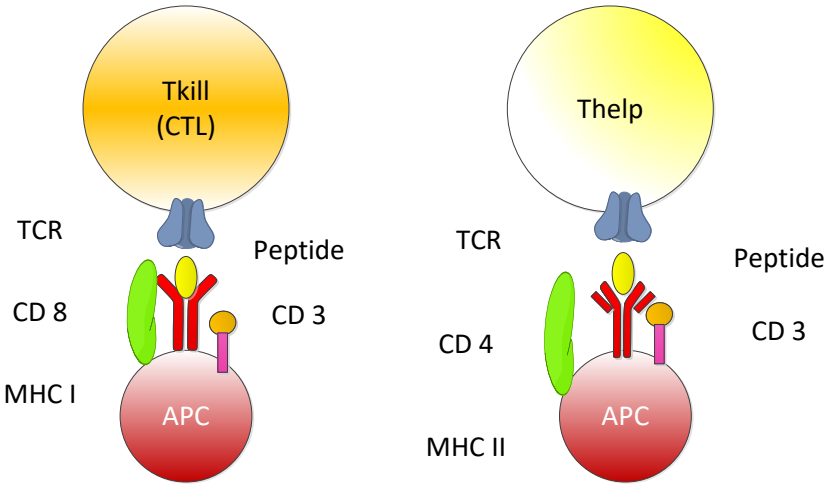
We detail the MHC I cell below with separate elements.



Now this is generated off of Chromosome 6 on the p region as depicted below.

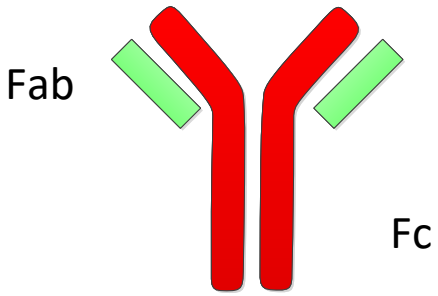


The two T cells, Tkill and Thelp are shown below. Tkill or the CTL have the ability to bind to MHC I and bind to CD-8 while the Thelp have the ability to bind to MHC II and bind to CD-4.

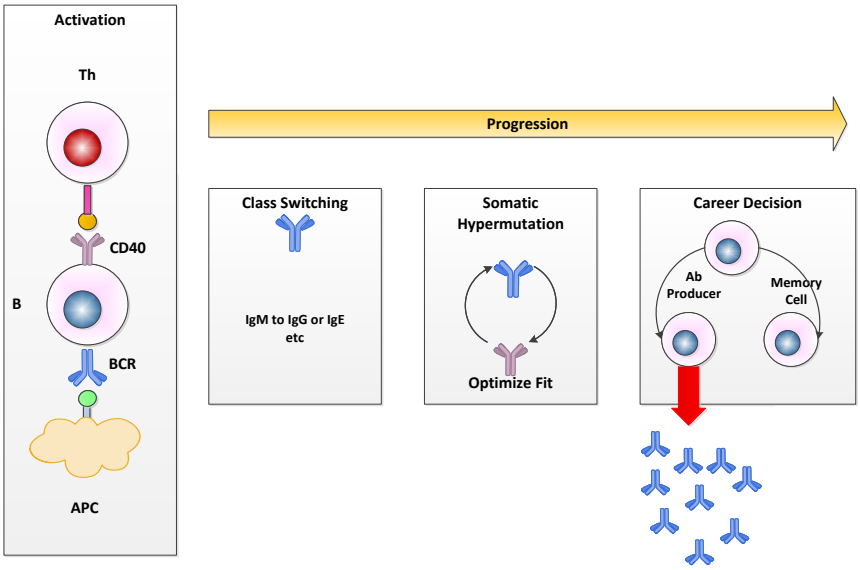


2.1.2 Ab Structure

Antibodies, Ab, and generated in the B cells. and they are protein structures with a long arm and a short arm. We shall not detail Ab structure here except to indicate its elements as Fab and Fc as shown below.

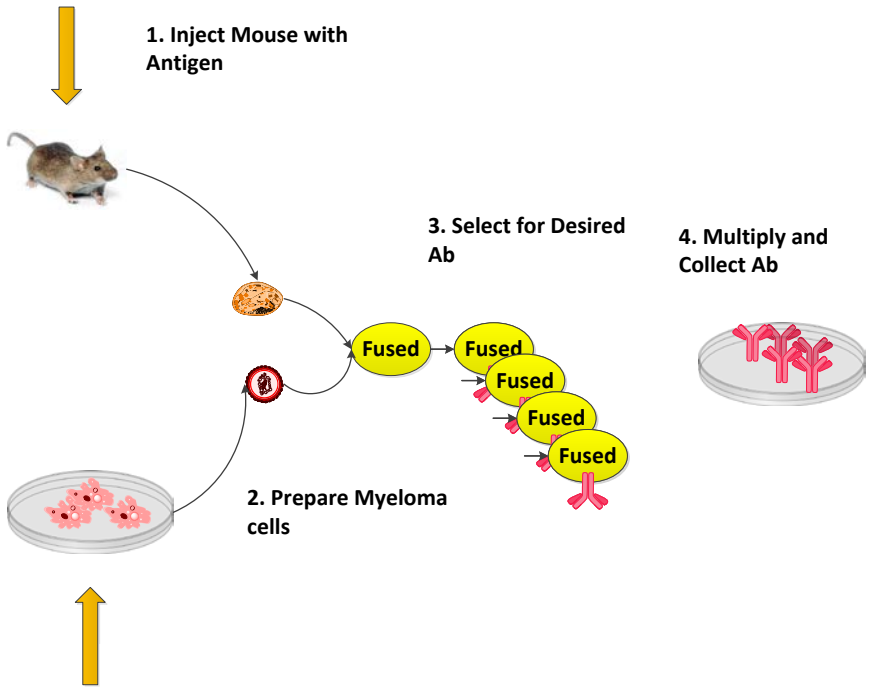


The antibodies generated in the B cells in response to an Ag can then go forth and attach to the targeted cell and commence the destruction process as shown in the Figure below.

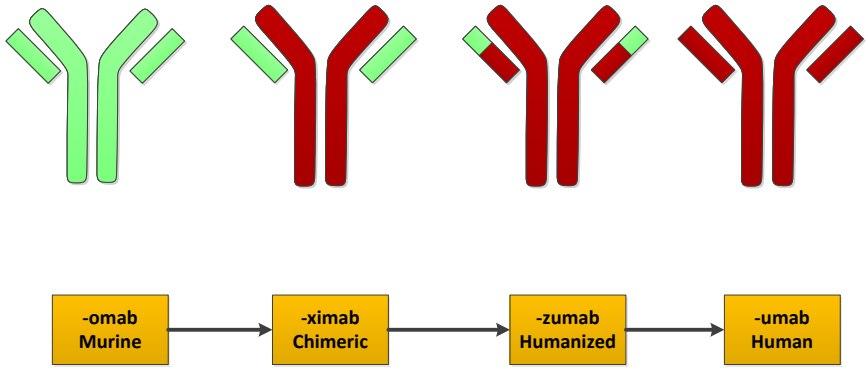


2.1.3 Monoclonal Antibodies

Monoclonal Ab can be made in what is comparatively a simple process. Namely we take a mouse, inject it with an antigen, then fuse the B cells from the mouse with Myeloma cells and then allow them to grow. They will produce Ab and one of these will be the one from the antigen, Ag. Then select that and we have a collection of these Ab. It may sound a bit simple but it is quite complex. We will detail this in the next chapter. But recall this is a murine Ab, not a human.



We then have to convert a murine to all human. The samples below depict some of the elements in this process.



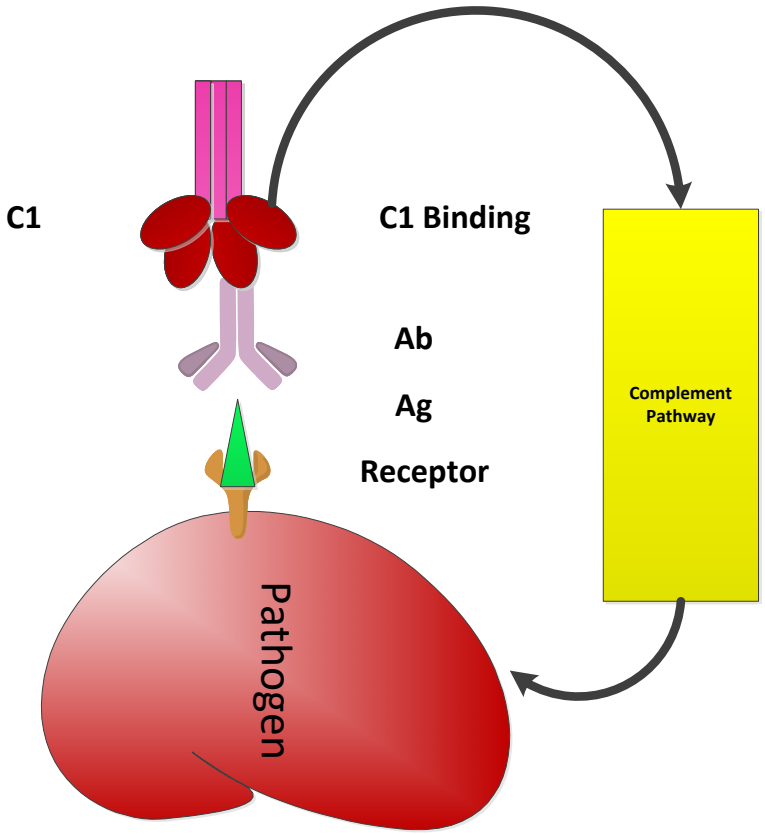
2.1.4 Antibody Mechanisms

The adaptive system works in a variety of ways. We present but a few here. Also, as one might expect, there may be a multitude of other mechanisms yet to be understood. This is typical as we better understand the functions of this complex system.

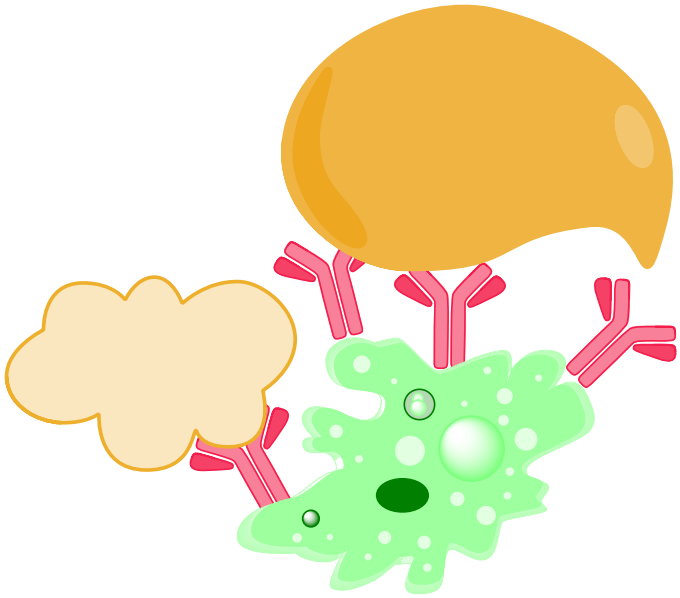
The Table below is a reiteration of what we saw earlier but now focusing on adaptive anti-body functioning. There are four presented but as noted, even now, there are variations of these as well.

	IgM, IgG, IgA			
Organisms	**Complement Activation**	**Opsonization**	**ADCC (Antibody Dependent Cell mediated Cytoxicity)**	**Neutralizing Antibody**
Viruses				
Bacteria (intracellular)				
Bacteria (extracellular)				
Protozoa (intracellular)				
Protozoa (extracellular)				
Fungi				
Flatworms				
Roundworms				

The first is the use of the complement system, that collection of proteins which release step after step until the target cell is destroyed.

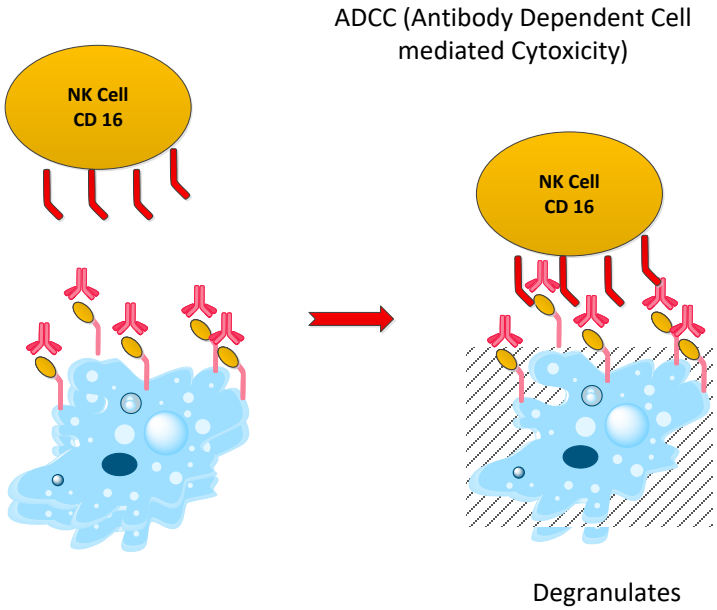


The second is the opsonization approach where classic phagocytes such as macrophages and even neutrophils get attached to a cell covered in antibodies and consume it.

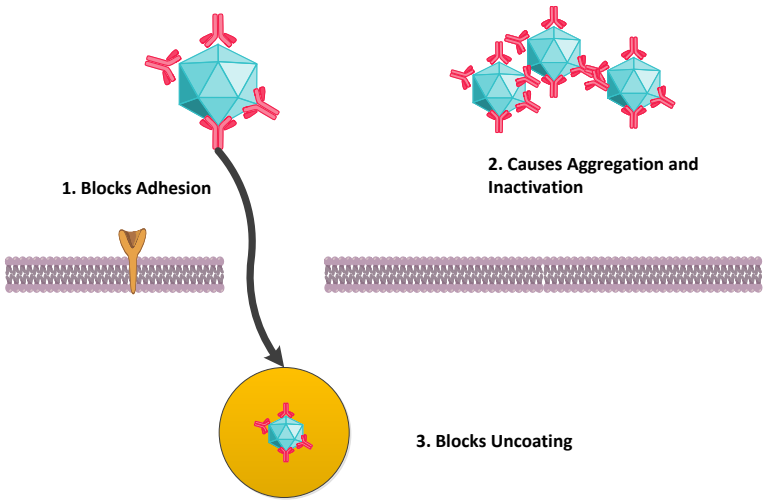


Opsonize: Phagocytes are bound to Ab and consume the pathogen

The third is the ADCC approach shown below where the Ab are used to isolate the pathogen, in this case a virus.



Finally, there is the neutralizing Ab approach where the Ab does not result in the killing but just in making the pathogen ineffective.



Neutralizing Antibodies NAb

These are the primary mechanisms. Clearly if one could target tumor cells in a similar fashion there is no reason these could be employed as well.

2.2 CIK

CIK are Cytokine Induce Killer Cells. They have been used to address several types of cancer and in a sense, are an amalgam approach.

As Jiang et al state:

Cytokine-induced killer (CIK) cells are a heterogeneous population of effector CD8 T cells with diverse TCR specificities, possessing non-MHC-restricted cytolytic activities against tumor cells. Therefore, CIK cells can lyse tumor cells in a non- MHC-restricted manner and can serve as an alternative cellular immunotherapy. This review summarizes technical aspects of CIK, current clinical experiences and future clinical utility.

Note here they describe it as an amalgam, the heterogeneous collection. They have various T Cell Receptors, TCR, and are non-MHC restrictive. They can be thought of as an enhanced brew of cytotoxic cells.

They continue:

The number of immune cells, especially dendritic cells and cytotoxic tumor infiltrating lymphocytes (TIL), particularly Th1 cells, CD8 T cells, and NK cells is associated with increased

survival of cancer patients. Such antitumor cellular immune responses can be greatly enhanced by adoptive transfer of activated type 1 lymphocytes.

Recently, adoptive cell therapy based on infusion of ex vivo expanded TILs has achieved substantial clinical success.

Cytokine-induced killer (CIK) cells are a heterogeneous population of effector CD8 T cells with diverse TCR specificities, possessing non-MHC-restricted cytolytic activities against tumor cells. Preclinical studies of CIK cells in murine tumor models demonstrate significant antitumor effects against a number of hematopoietic and solid tumors. Clinical studies have confirmed benefit and safety of CIK cell-based therapy for patients with comparable malignancies. Enhancing the potency and specificity of CIK therapy via immunological and genetic engineering approaches and identifying robust biomarkers of response will significantly improve this therapy.

The above description affirms the CD8 T cell elements but they may also contain NK cells as well.

Schmeel et al note:

Cytokine-induced killer (CIK) cells are a heterogeneous population of immune effector cells that feature a mixed T- and Natural killer (NK) cell-like phenotype in their terminally-differentiated CD3+CD56+ subset. The easy availability, high proliferation rate and widely major histocompatibility complex (MHC)-unrestricted antitumor activity of CIK cells contribute to their particularly advantageous profile, making them an attractive approach for adoptive immunotherapy.

CIK cells have shown considerable cytotoxicity against both solid tumors and hematological malignancies in vitro and in animal studies. Recently, initial clinical experiences demonstrated the feasibility and efficacy of CIK cell immunotherapy in cancer patients, even at advanced disease stages. Likewise, the clinical application of CIK cells in combination with standard therapeutic procedures revealed synergistic antitumor effects. In this report, we will focus our consideration on CIK cells in the treatment of hematological malignancies. We will give insight into the latest advances and future perspectives and outline the most prominent results obtained in 17 clinical studies.

Overall, CIK cells demonstrated a crucial impact on the treatment of patients with hematological malignancies, as evidenced by complete remissions, prolonged survival durations and improved quality of life. However, up to now, the optimal application schedule eventually favoring their integration into clinical practice has still to be developed.

As Guo and Han state:

Cytokine-induced killer (CIK) cells are a heterogeneous cell population that was first discovered in the 1990s and can be generated from lymphocytes co-cultured with an anti-CD3 antibody and many other cytokines in vitro. Numerous studies have demonstrated that CIK cells exhibit active proliferation and potent antitumor cytotoxicity against multifarious tumor cells in vitro and in

vivo. Increasing data show that the antitumor effects of CIK cells rely on a perforin-based mechanism and Fas-Fas ligand interactions. CIK cells are also not inhibited by immunosuppressive drugs, which makes CIK cells an ideal candidate cell type for cancer therapy.

Theoretically, CIK cell-based adoptive cellular immunotherapy (ACI) could be a curative strategy for cancer. Abundant clinical trials on this therapeutic regimen have been published in the past two decades, confirming its safety and feasibility in cancer patients [6-8]. Several other clinical trials focusing on graft-versus-host disease (GVHD) and viral infections related to this therapy have also been conducted in recent years. Given the ongoing investigations of CIK cell-based ACI, this regimen has potentially widespread application prospects in the clinic for most types of cancer.

In addition, several strategies to improve the clinical effects of CIK cells have been conducted. For example, CIK cells combined with traditional cancer treatments, including surgery, chemotherapy, and radiotherapy, may achieve the best objective responses in patients. Furthermore, preconditioning chemotherapy, activated cytokines, and specific antibodies could enhance the antitumor ability of CIK cells ….

Up to now, intensive and strict studies on the immune phenotype of CIK cells have been conducted. CIK cells, which are a heterogeneous cell population, comprise CD3+CD56+, CD3+ CD56−, and CD3− CD56+ cells [18]. CD3+CD56+ cells, which are derived from CD3+CD56− T cells, are also called natural killer T (NKT) cells and are primarily responsible for non-major histocompatibility complex (MHC)-restricted antitumor activity [19,20]. Furthermore, this subset co-expresses CD2, T-cell receptor (TCR) αβ, and CD8, but not CD16 [21].

In addition, CD3+CD56+ cells bear the CD27+CD28− or CD27−CD28− phenotype because they belong to terminally differentiated T-cell populations, whereas CD3+CD56− cells are subjected to early differentiation and mainly express the CD27+CD28+ and CD62L+ phenotypes [22,23]. Meanwhile, CD3+CD56− cells also express CD4, CD8, and TCRαβ [21,23]. CD3−CD56+ cells behave similarly to conventional natural killer (NK) cells and express classical NK-cell receptors [23]. In addition to these markers, CIK cells express CD45RA, C-C chemokine receptor (CCR)7, CD11a, macrophage inflammatory protein 1a, perforin, and Fas ligand…

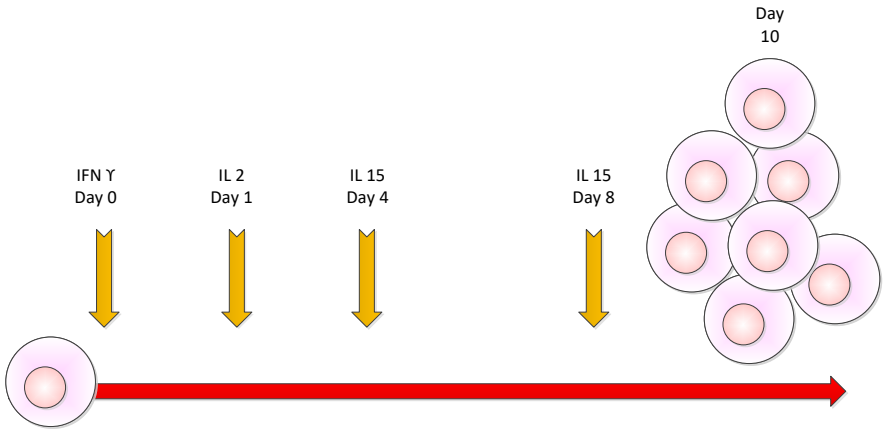
The following continuation regarding the use of CIK is extremely important. GVHD is oftentimes a severe response to immune-treatments. It appears that CIKs mitigate this effect.

Allogeneic transplantation is a common treatment for hematopoietic malignancies, and GVHD, a life-threatening symptom of allogeneic transplantation, is a main cause of transplantation-related death. Because of the extensive research and application of CIK cell therapy for hematologic diseases, we separately listed and discussed the relationship between CIK cells and GVHD. Moreover, traditional immune-suppressants frequently fail to suppress GVHD; thus, a safe and effective approach is needed to better treat patients with GVHD.

Recent studies have demonstrated that NKT cells can suppress transplant rejection [39,40]. Therefore, the anti-GVHD properties of CIK cells are similar to those of NKT cells because of CD3 +CD56+ CIK cells, here referred to as NKT cells.

Although hematopoietic stem cell transplantation (HSCT) is an effective treatment for hematologic malignancies, allogeneic immune activation might promote GVHD. With the transplantation of CIK cells after HSCT, the risk of GVHD decreases. Based on the information above, CIK cell therapy is regarded as an effective immunosuppressant of GVHD, which often occurs following allogeneic transplantation.

Now from the above the following is a graphical description of the development of CIK cells. Namely one starts with some ex-vivo cells from the patient and then over a period of say ten days enhance them with Interferon and Interleukins. Then at the conclusion reinfuse the developed mass into the patient.

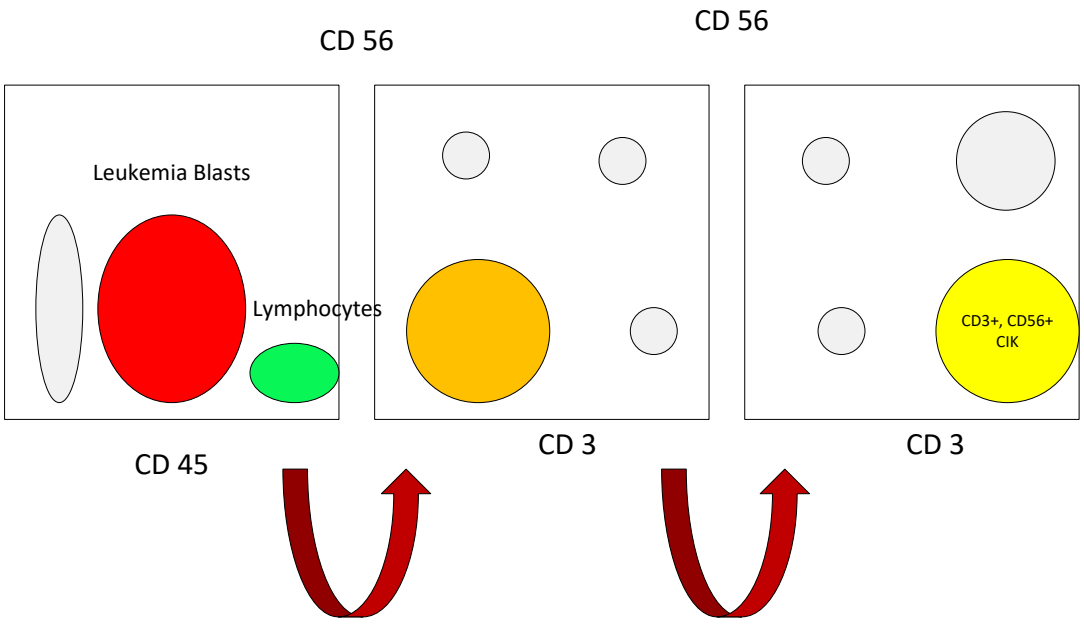


APC: allophycocyanin; APC-A700: APC-Alexa Fluor 700; APC-A750: APC-Alexa Fluor 750; CIK: cytokine-induced killer; CsA: Ciclosporin A; FITC: fluorescein– isothiocyanate; ECD: phycoerythrin-Texas Red®; GvL/T: graft-versus-leukemia/ tumor; DLI: donor lymphocyte infusion; GvHD: graft-versus-host disease; GMP: good manufacturing practice; HSCT: hematopoietic stem cell transplantation; KO: krome orange; MMF: mycophenolate mofetil; MPA: mycophenolic acid; MRD: minimal residual disease; NK: natural killer; PBMC: peripheral blood mononuclear cells; PB: pacific blue™; PC-5: phycoerythrin–cyanine-5; PC-7: phycoerythrin–cyanine-7; PE: phycoerythrin. See: Bremm *et al. J Transl Med (2016) 14:264*

Furthermore, we can look at the cells after processing and see a collection of CD enhanced profiles, taking the result from the initial profile dominated by the malignant cells. What essentially CIKs are doing is trying to enhance the limited immunity still present in the patient and then reinfusing a specific profile of those back into the patient. It is a means of boosting the immune system of the patient themselves. Thus, the reduction of GVHD.

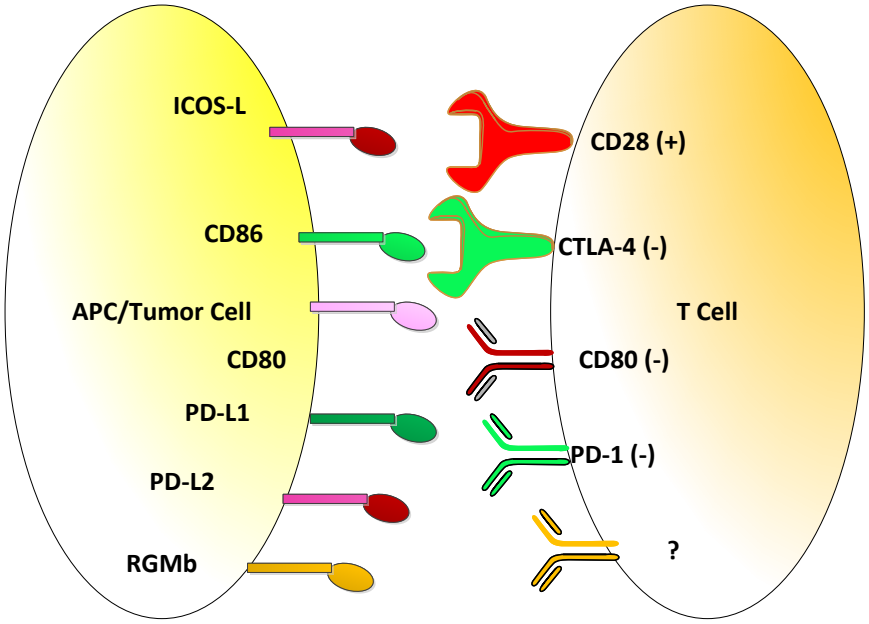
Marrow cells obtained from newly diagnosed AML samples analyzed by side scatter versus CD45 staining show a large proportion of leukemic blasts that are CD 45 dim and a much smaller fraction of normal lymphocytes that are CD 45 bright (a). Culture of the bulk marrow cells comprising of majority of leukemic blasts with a small fraction of lymphocytes under CIK condition (b) is able to generate an end product comprising of a majority of CD3+ cells with a variable CD3+CD56+ fraction (c).

Linn and Hui, Journal of Biomedicine and Biotechnology Volume 2010, Article ID 435745



2.3 Receptors: Checkpoint Inhibitors

The above characterizations of the operation of the immune system is but a first step. There are a multiplicity of ligands and receptors which can enhance the process or inhibit the process. The inhibitory one are checkpoints and therapy addressing these inhibitory functions are termed checkpoint blockade. We briefly depict some of the current and somewhat well-known ones. A warning should be noted. It seems to be common place amongst immune therapies that as one barrier is climbed others soon appear. Thus, this may very well be merely a first step in an ever continuing understanding of the complexities of the immune systems.



2.3.1 CTLA-4

CTLA-4 is a checkpoint inhibitor. It has the potential to inhibit the actions of the immune cells to the cell expression this. As Topalian et al state:

The conventional wisdom underlying our vision of how CTLA-4 blockade mediates tumor regression is that it systemically activates T cells that are encountering antigens.

CTLA-4 represents the paradigm for regulatory feedback inhibition. Its engagement down-modulates the amplitude of T cell responses, largely by inhibiting co-stimulation by CD28, with which it shares the ligands CD80 and CD86. As a ‘‘master T cell co-stimulator,’’ CD28 engagement amplifies TCR signaling when the T cell receptor (TCR) is also engaged by cognate peptide-major histocompatibility complex (MHC).

However, CTLA-4 has a much higher affinity for both CD80 and CD86 compared with CD28, so its expression on activated T cells dampens CD28 co-stimulation by out-competing CD28 binding and, possibly, also via depletion of CD80 and CD86 via ‘‘trans-endocytosis’’. Because CD80 and CD86 are expressed on antigen-presenting cells (APCs; e.g., dendritic cells and monocytes) but not on non-hematologic tumor cells, CTLA-4’s suppression of anti-tumor immunity has been viewed to reside primarily in secondary lymphoid organs where T cell activation occurs rather than within the tumor microenvironment (TME).

Furthermore, CTLA-4 is predominantly expressed on CD4+ ‘‘helper’’ and not CD8+ ‘‘killer’’ T cells. Therefore, heightened CD8 responses in anti-CTLA-4-treated patients likely occur indirectly through increased activation of CD4+ cells. Of note, a few studies suggest that CTLA-4 can act as a direct inhibitory receptor of CD8 T cells, although this role in down-modulating anti-tumor CD8 T cell responses remains to be directly demonstrated. The specific signaling pathways by which CTLA-4 inhibits T cell activation are still under investigation, although

activation of the phosphatases SHP2 and PP2A appears to be important in counteracting both tyrosine and serine/threonine kinase signals induced by TCR and CD28.

CTLA-4 engagement also interferes with the ‘‘TCR stop signal,’’ which maintains the immunological synapse long enough for extended or serial interactions between TCR and its peptide-MHC ligand. Naive and resting memory T cells express CD28, but not CTLA-4, on the cell surface, allowing costimulation to dominate upon antigen recognition.

2.3.2 PD-1

In a similar manner to CTLA-4, PD-1 is also an inhibitor. As Topalian et al state:

The PD-1 system of immune modulation bears similarities to CTLA-4 as well as key distinctions. Similar to CTLA-4, PD-1 is absent on resting naive and memory T cells and is expressed upon TCR engagement. However, in contrast to CTLA-4, PD-1 expression on the surface of activated T cells requires transcriptional activation and is therefore delayed.

Also in contrast to CTLA-4, PD-1 contains a conventional immunoreceptor tyrosine inhibitory motif (ITIM) as well as an immunoreceptor tyrosine switch motif (ITSM). PD-1's ITIM and ITSM bind the inhibitory phosphatase SHP-2. PD-1 engagement can also activate the inhibitory phosphatase PP2A. PD-1 engagement directly inhibits TCR-mediated effector functions and increases T cell migration within tissues, thereby limiting the time that a T cell has to survey the surface of interacting cells for the presence of cognate peptide-MHC complexes.

Therefore, T cells may ‘‘pass over’’ target cells expressing lower levels of peptide-MHC complexes. In contrast to CTLA-4, PD-1 blockade is viewed to work predominantly within the TME, where its ligands are commonly overexpressed by tumor cells as well as infiltrating leukocytes. This mechanism is thought to reflect its important physiologic role in restraining collateral tissue damage during T cell responses to infection. In addition, tumor-infiltrating lymphocytes (TILs) commonly express heightened levels of PD-1 and are thought to be ‘‘exhausted’’ because of chronic stimulation by tumor antigens, analogous to the exhausted phenotype seen in murine models of chronic viral infection, which is partially reversible by PD-1 pathway blockade.

Importantly, the phenotypes of murine knockouts of PD-1 and its two known ligands are very mild, consisting of late-onset organ-specific inflammation, particularly when crossed to autoimmune- prone mouse strains. This contrasts sharply with the Ctla-4 knockout phenotype and highlights the importance of the PD-1 pathway in restricting peripheral tissue inflammation. Furthermore, it is consistent with clinical observations that autoimmune side effects of anti-PD-1 drugs are generally milder and less frequent than with anti- CTLA-4. Despite the conventional wisdom that CTLA-4 acts early in T cell activation in secondary lymphoid tissues whereas PD-1 inhibits execution of effector T cell responses in tissue and tumors, this distinction is not absolute.

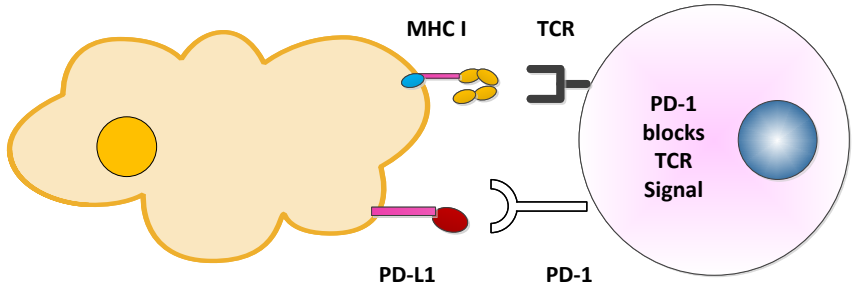
Beyond its role in dampening activation of effector T cells, CTLA-4 plays a major role in driving the suppressive function of T regulatory (Treg) cells. Tregs, which broadly inhibit effector T cell

responses, are typically concentrated in tumor tissues and are thought to locally inhibit anti-tumor immunity.

Therefore, CTLA-4 blockade may affect intratumoral immune responses by inactivating tumor-infiltrating Treg cells. Recent evidence has demonstrated anti-tumor effects from CTLA-4 blockade even when S1P inhibitors block lymphocyte egress from lymph nodes, indicating that this checkpoint exerts at least some effects directly in the TME as opposed to secondary lymphoid tissues.

Conversely, PD-1 has been shown to play a role in early fate decisions of T cells recognizing antigens presented in the lymph node. In particular, PD-1 engagement limits the initial ‘‘burst size’’ of T cells upon antigen exposure and can partially convert T cell tolerance induction to effector differentiation.

The authors present a graphic regarding how this blocking or checkpoint functions. We depict this below.



Oncogenic pathway in tumor cell uses AKT for gene amplification allowing PD-L1 expression

As Freeman states:

T cell activation requires a TCR mediated signal, but the strength, course, and duration are directed by costimulatory molecules and cytokines from the antigen-presenting cell (APC). An unexpected finding was that some molecular pairs attenuate the strength of the TCR signal, a process termed coinhibition. The threshold for the initiation of an immune response is set very high, with a requirement for both antigen recognition and costimulatory signals from innate immune recognition of ‘‘danger’’ signals.

Paradoxically, T cell activation also induces expression of coinhibitory receptors such as programmed death-1 (PD-1). Cytokines produced after T cell activation such as INF- and IL-4 up-regulate PD-1 ligands, establishing a feedback loop that attenuates immune responses and limits the extent of immune-mediated tissue damage unless overridden by strong costimulatory signals. PD-1 is a CD28 family member expressed on activated T cells, B cells, and myeloid cells. In proximity to the TCR signaling complex, PD-1 delivers a coinhibitory signal upon binding to either of its two ligands, PD-L1 or PD-L2.

Engagement of ligand results in tyrosine phosphorylation of the PD-1 cytoplasmic domain and recruitment of phosphatases, particularly SHP2. This results in dephosphorylation of TCR proximal signaling molecules including ZAP70, PKC, and CD3, leading to attenuation of the TCR/CD28 signal.

The role of the PD-1 pathway in peripheral T cell tolerance and its role in immune evasion by tumors and chronic infections make the PD-1 pathway a promising therapeutic target.

2.3.3 KIR

As Topalian et al state:

NK cells are a population of innate immune cells with well documented roles in infectious and tumor immunity. Like activated CD8 T cells, NK cells mediate target cell apoptosis via secretion of preformed granules containing perforin and granzymes. However, unlike CD8 T cells, NK cells do not recognize unique peptides in the context of classical MHC I molecules.

Instead, NK function is controlled by the complex interplay of a series of activating receptors and killer inhibitory receptors (KIRs) and their ligands. In humans, KIR molecules are polymorphic and bind to certain MHC I alleles, and not all KIR/ ligand pairs are equally capable of inhibiting NK cell function.

Indeed, bone marrow transplants in which donor NK cells lack the ability to be inhibited by host KIR ligands have been shown to result in lower relapse rates and improved OS, supporting the importance of this cell type in cancer immunity. The relative importance of NK cells in murine models of cancer immunotherapy has been documented by multiple studies but is especially highlighted by studies in which NK cell activation via IL-15 can eradicate fairly advanced tumors in the absence of CD8 T cells. So, in a sense, KIRs can be thought of as immune checkpoint molecules, and blocking KIRs on NK cells could be exploited to augment anti-tumor immunity.

To that end, a fully human anti-KIR mAb has entered clinical testing. This antibody (initially IPH-2101, Innate Pharma; now lirilumab, Bristol-Myers Squibb) binds to the human KIR molecules KIR2DL-1, KIR2DL-2, and KIR2DL-3 as well as to KIR2DS-1 and KIR2DA-2, preventing their binding to HLA-C MHC I molecules. A phase I trial of anti-KIR in acute myelogenous leukemia has been completed. Several studies in hematologic and solid cancers are ongoing, but of particular interest are trials in which lirilumab is being combined with anti-PD-1 (nivolumab) or with anti-CTLA-4 (ipilimumab). These trials are important in that each seeks to combine innate immune activation via anti-KIR with activation of the adaptive immune system, therefore offering the potential for additive or synergistic anti-tumor efficacy.

2.3.4 Toll Like Receptors

The Toll Like Receptors, "toll" means weird or strange in German, and they play a significant role in the innate system.

As Travis notes:

At the heart of this protection are proteins, called Toll-like receptors (TLRs), on cells of the innate immune system. Over the past decade, it has become clear that TLRs are the long-sought cell-surface receptors that recognize common microbial features such as bacterial wall components or the distinctive DNA sequences of a virus. This role could date back to the earliest multicellular organisms, as humans and some of the most evolutionarily primitive animals share TLRs and the molecules involved in the TLR signaling cascade.

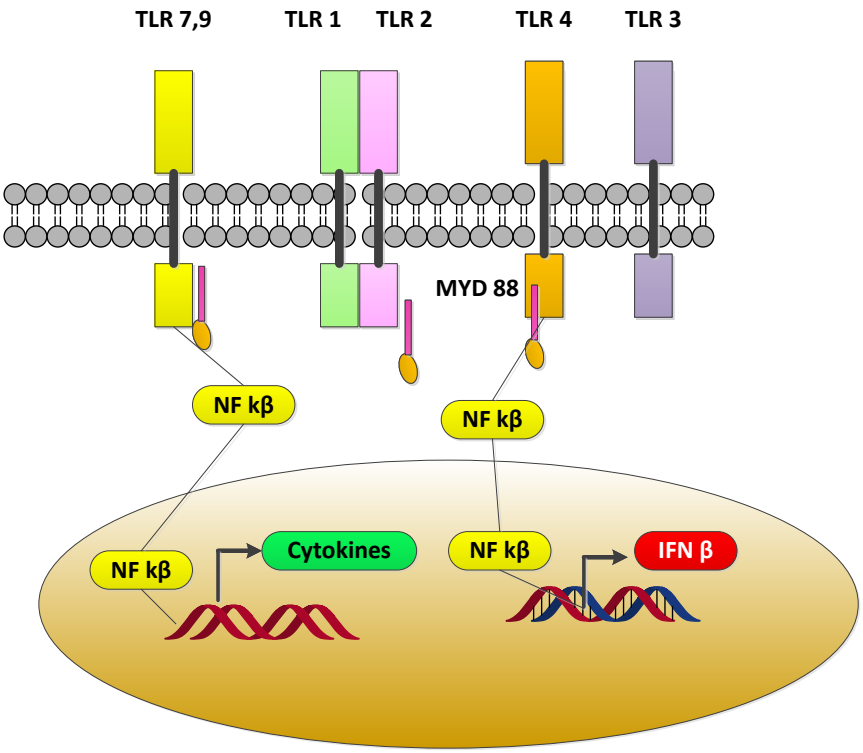
Takeda and Ashira note:

Toll receptor was originally identified in Drosophila as an essential receptor for the establishment of the dorso-ventral pattern in developing embryos [1]. In 1996, Hoffmann and colleagues demonstrated that Toll-mutant flies were highly susceptible to fungal infection [2]. This study made us aware that the immune system, particularly the innate immune system, has a skillful means of detecting invasion by microorganisms.

Subsequently, mammalian homologues of Toll receptor were identified one after another, and designated as Toll-like receptors (TLRs). Functional analysis of mammalian TLRs has revealed that they recognize specific patterns of microbial components that are conserved among pathogens, but are not found in mammals. In signaling pathways via TLRs, a common adaptor, MyD88, was first characterized as an essential component for the activation of innate immunity by all the TLRs.

However, accumulating evidence indicates that individual TLRs exhibit specific responses. Furthermore, they have their own signaling molecules to manifest these specific responses. In this review, we will focus on the recent advances in our understanding of the mechanism of TLR-mediated signaling pathways.

Now following their analysis, we can depict the TLR functions as shown below.

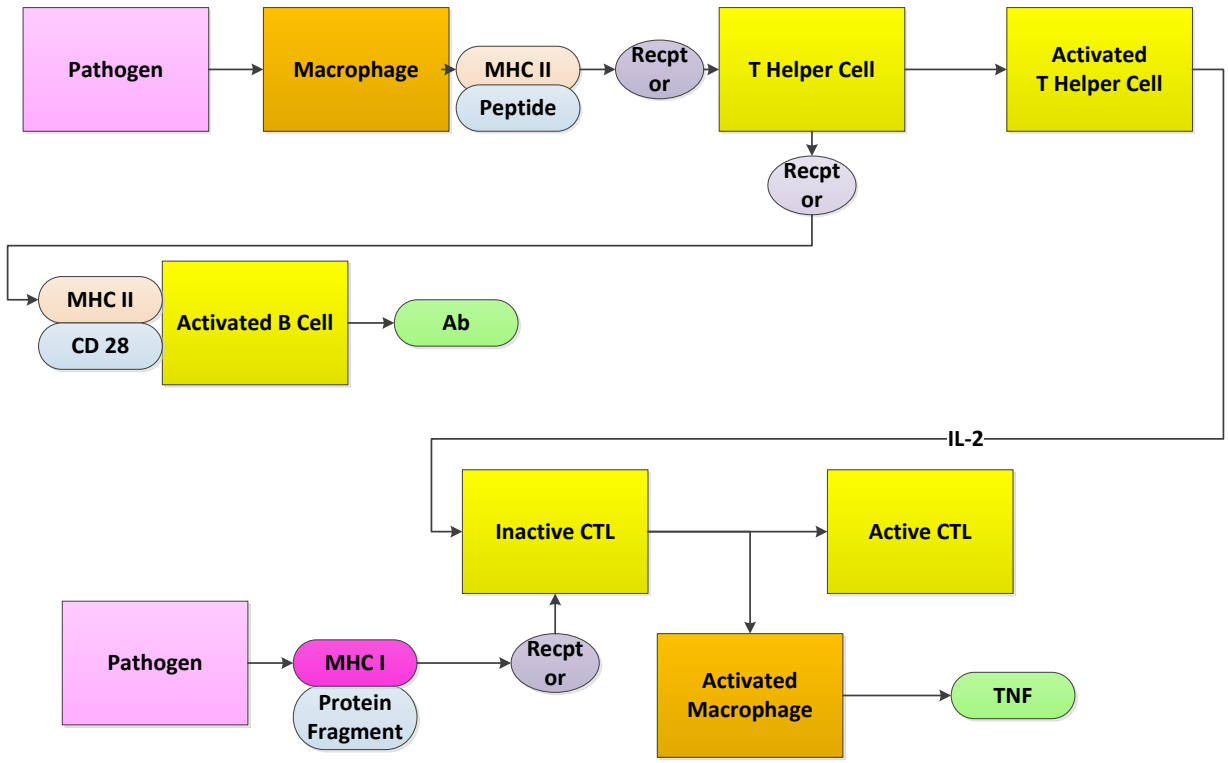


We will see more from these TLR as we proceed.

2.4 The Immune System as Process

The following is, at a very high level, a complete set of interactions of the immune system. We have not addressed specific receptors nor have we detailed control mechanism. But generally, the system functions as described below. The innate and the adaptive systems function somewhat hand in glove and then what we have below is primarily the adaptive system.

Pathogens activate the system and depending on where and how they are initiated various elements take over. The checkpoints we described previously can arise and inhibit this process. The checkpoints arise as a result of intracellular pathway aberrations. These aberrations can be met by immunotherapeutic approaches and/or by therapeutics dealing with the pathway itself. Melanoma therapeutics is an example of this approach.



This simplified diagram above depicts a high-level understanding of the adaptive immune system. The key observation in this section is not just the high-level elements, but as we noted with checkpoints, the ever-evolving complexities that throttle the immune system.

The importance of this diagram is that like so many models of gene interaction in cancer cells, this is a model of immune system interaction. It is in a simple manner the beginning os an engineering approach to understanding and utilizing the immune system. It combines the grammar, namely the differing elements, and the logic, how these elements interplay to effect something. The rhetorical side, namely applying these to address a pathology, is what we will examine next.

3 IMMUNE SYSTEM DYNAMICS: SET POINTS

There has been a multiplicity of applications of such therapeutics as monoclonal antibodies which are used as "checkpoint inhibitors" to treat a variety of cancers. These therapeutics are proteins which can block the action of inhibitors on T cells which if activated and prevent the T cell from attacking the cancer cell. We have seen the proliferation of these therapeutic approaches in melanoma, lung cancer and even prostate cancer.

The main driver for this analysis is the recent work of Chen and Mellman. In a recent paper these two authors state:

*Immunotherapy is proving to be an effective therapeutic approach in a variety of cancers. But despite the clinical success of antibodies against the immune regulators CTLA4 and PD-L1/PD-1, only a subset of people exhibit durable responses, suggesting that a broader view of cancer immunity is required. Immunity is influenced by a complex set of tumour, host and environmental factors that govern the strength and timing of the anticancer response. Clinical studies are beginning to define these factors as immune profiles that can predict responses to immunotherapy. In the context of the cancer immunity cycle, such factors combine to represent the inherent immunological status — or '**cancer–immune set point'** — of an individual.*

The concept of a "set point" is in our opinion rather poorly used. The construct, if properly understood, means that there is some point at which T cell activators and inhibitors either permit activation and effective T cell immunotherapeutic action or inhibit that. Namely there is some set of activations less inhibitions which all T cells to perform and under that "set point" they no longer function.

If such a concept has physical meaning, then the authors state:

Although largely conceptual, the idea of a set point provides a framework to help organize the torrent of clinical and biomarker data that will emerge over the coming months and years. The number of targets that could prove effective for cancer immunotherapy is great; the number of potential combinations of therapeutic agents that are directed against these targets (or combinations of such agents with conventional standard-of-care agents) is even greater. The development of some cancer therapies may be largely empirical, but it can be guided by considering, even in general terms, the elements that comprise cancer immunity

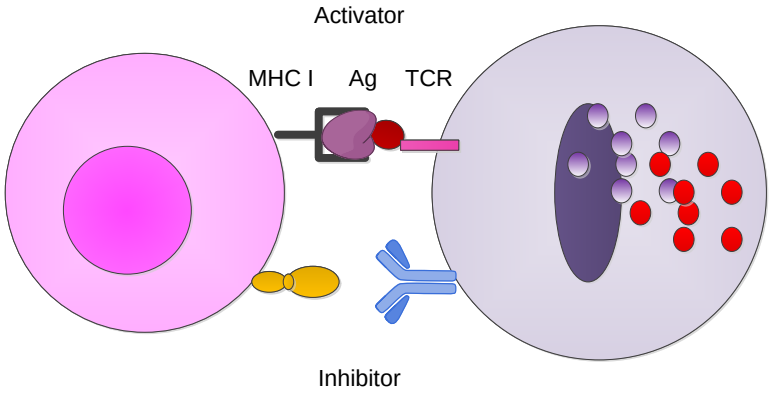
Thus, our objective in this paper is to examine this set point concept and explore its dimensions. Specifically, we examine how it may be used for therapeutic uses.

3.1 SOME PRINCIPLES

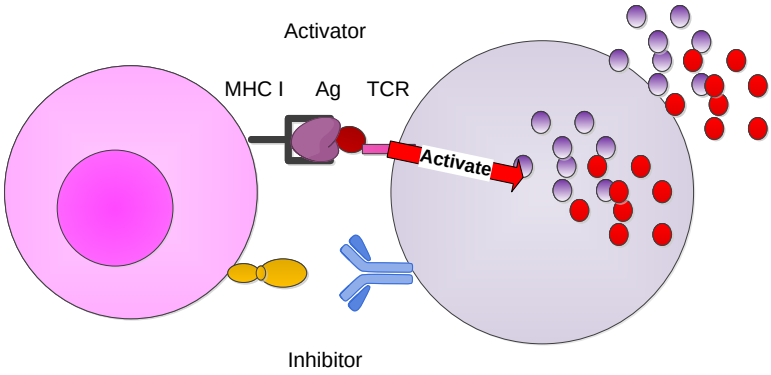
To best understand some of the principles we examine some simplistic model.

3.1.1 Activator/Inhibitors

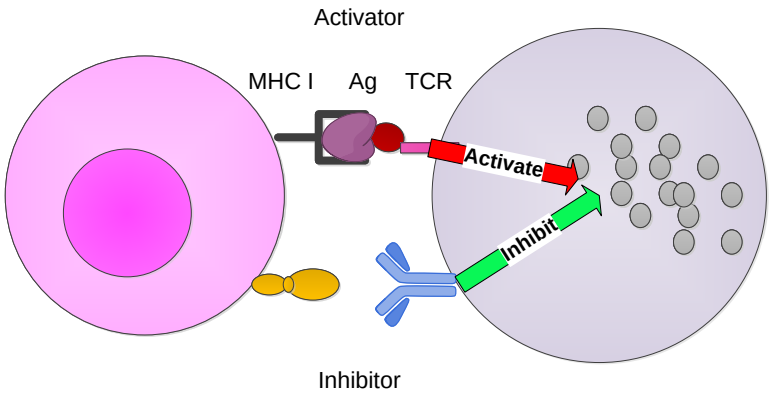
Let us begin with a simplistic but representative set of examples. We know that a CD8 T cell has a receptor, the TCR, T cell receptor, and it examines an antigen presenting cell, APV, which presents an antigen on its MHC I protein. This process is essentially an activator process. If that were all which was needed, then the T cell would be activated and sent out its cytokines and destroy the cell. However, there are also inhibitor ligands which activate inhibition in the T cell. We show these two below.



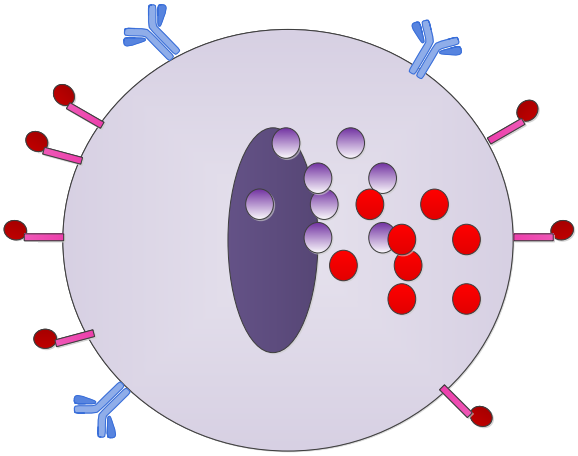
Now if the T cell is activated and the inhibition is not then we get the T cell sending out cytokines and killing the offending cell.



However if the T cell has an inhibitor also connected then the inhibitor sends out an internal T cell signal which stops the release. The presenting cell survives.



Now in reality the T cells do not have just one receptor. It may have a multiplicity over the surface. Thus there are a multiplicity of activators and inhibitors. It is a multiplicity amongst the same type as well a multiplicity of types. In fact the T cell may be just covered with receptors searching out antigens.



Thus the first layer of complexity of the immune response is not a simple activator/inhibitor complex but a mass of receptors and ligands interacting in a complex manner. The question then is; at what point does the T cell go from active to inactive and back again? In fact we may ask if there is some hysteresis effect. If so can we therapeutically take advantage of it.

3.1.2 Ligand/Receptor Dynamics

Ligands and receptors are basically two separate chemical elements. The binding of them is also essentially a chemical process whereby the ligand finds the correct location on the receptor to bind. It is in many ways like any chemical reaction. As such there is a reaction rate whereby the ligand and receptor combine, but equally there is the reverse reaction, the breaking apart of a bond.

In the work by Stone et al the authors note:

The interaction between the T-cell receptor (TCR) and its peptide–major histocompatibility complex (pepMHC) ligand plays a critical role in determining the activity and specificity of the T cell. The binding properties associated with these interactions have now been studied in many systems, providing a framework for a mechanistic understanding of the initial events that govern T-cell function. There have been various other reviews that have described the structural and biochemical features of TCR: pepMHC interactions.

Here we provide an overview of four areas that directly impact our understanding of T-cell function, as viewed from the perspective of the TCR: pepMHC interaction:

(1) relationships between T-cell activity and TCR: pepMHC binding parameters,

(2) TCR affinity, avidity and clustering,

(3) influence of coreceptors on pepMHC binding by TCRs and T-cell activity, and

(4) impact of TCR binding affinity on antigenic peptide specificity.

Namely there is a reaction such as:

$$TCR + pepMHC \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} TCR : pepMHC$$

Now they conclude:

The binding properties of TCRs for their pepMHC ligands are critically important in the function of T cells, leading to outcomes that can involve T-cell selection in the thymus or full peripheral T-cell responsiveness or homeostatic T-cell proliferation in the periphery. The processes are even more complicated because the same TCR could interact with multiple pepMHC ligands on the same antigen-presenting cell, each with heterogeneous binding properties. These reactions would result in a complex integration of signals that ultimately determine the nature of the T-cell response.

While there have been numerous studies to elucidate the precise binding parameters that correlate with different T-cell activities, various questions remain unanswered (in part because of the technical difficulties associated with performing binding experiments on low-affinity reactions). Further understanding of the TCR binding properties that generate defined signals is important, not only from a basic science perspective but also toward developing optimal strategies that improve T-cell responses to foreign antigens and tumour antigens.

Thus, one must be careful in developing an immune set point theory to be cautious about the affinity issues as discussed above.

3.1.3 Overall Process

We have examined the complex process fundamentally as a build. Specifically:

1. Activation: When an antibody binds with the TCR we expect a response.

2. Inhibitor: When there is an inhibitor, however, it may be possible to block the pathway leading to the activation.

3. Notwithstanding the above, the cell actually has a multiplicity of the previous two and thus there may be some race with a finish line defined by what has been called a "set point", or simply some collection of activators and inhibitors seeing which one dominates.

4. There are not just one possible activator and inhibitor. For a T cell we have the TCR but we may have well more than a PD-1. New inhibitors are arising each day.

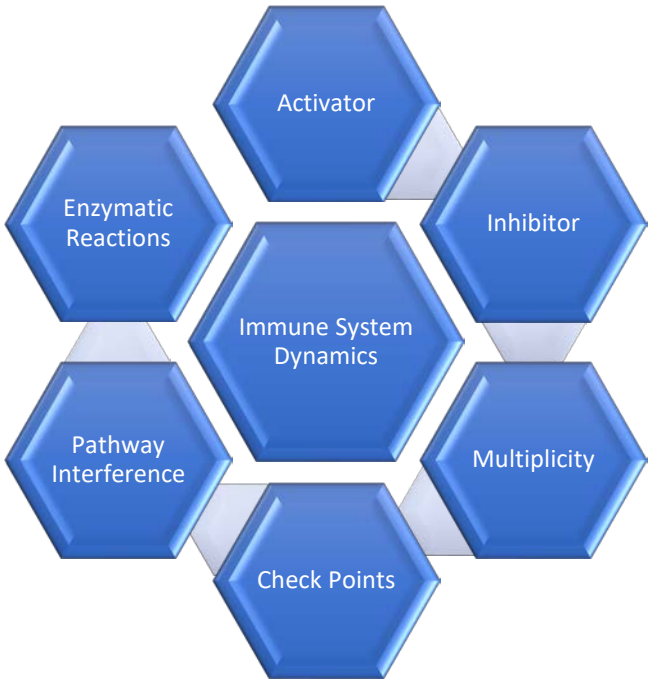
5. The internal machinations of the cellular pathways may also effect the net result. Thus, genetic changes can affect what happens.

6. The kinetics of the binding can and often do play a significant role. Binding is not a one-way street, and the result may be loss of tumor control.

7. Exogeneous Factors: The human biome is often a driving factor to the efficacy of immunotherapy. As Chen and Mellman note:

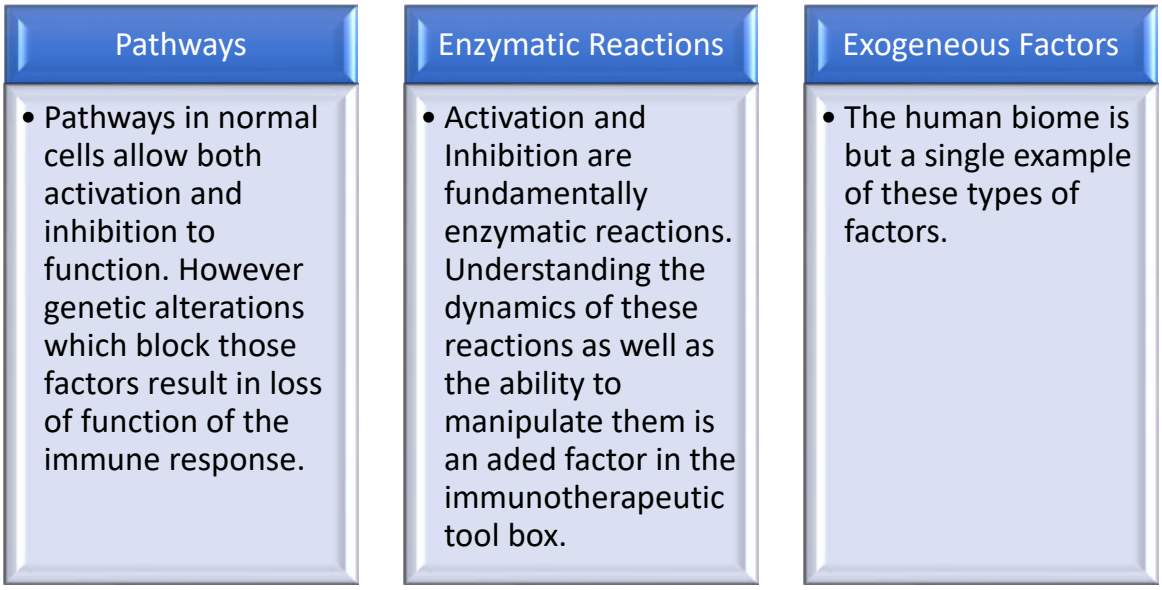
Factors that are extrinsic to the tumour or host genomes may also affect the immune profile of tumors. Chief among these is the gut microbiome, which has an important role not only in influencing the initiation of some cancers, but also in the response to chemotherapy and immunotherapy…mice bearing subcutaneous syngeneic tumors do not respond to chemotherapy if sterilized by prior treatment with antibiotics or when raised in germ-free conditions. The effect was attributed to the ability of commensal bacteria to activate the innate immune system of the host following chemotherapy, possibly by causing symbiosis and penetration of commensal bacteria into the gut lamina propria.

Subsequent work established an even clearer link between T-cell responses and an intact microbiota1. Fecal transfer or co-housing experiments in mice demonstrated that defined species of gut bacteria enabled antitumor responses after treatment with anti-PD-L1/PD-1 or anti-CTLA4 therapies. Furthermore, the gut microbiota even influenced spontaneous antitumor responses, which correlated with the degree of T-cell infiltration into tumors before any therapy had been administered.



Each of these elements can be considered as a therapeutic target for immunotherapy. We summarize some of these below.

Activator	Inhibitot	Multiplicity	Checkpoints
•Activators start the process of making the immune system attack the putative pathogen	•Inhibitors are often a "self" determinator yet are used by cancer cells to neutralize the immune system.	•The receptors are spread all across the surface of a cell. Also there is a multiplicity of different receptors. Thus, multiplicity has two meanings: • (i) multiplicity of a single type on a single cell, and • (ii) a multiplicity of different types on a single cell. •This is both a complexity and an opportunuity for immunotherapeutic targets.	•Checkpoints are an extension of inhibitors and hev become a key factor in current immunotherapy.



Thus, understanding the specifics may be a useful approach in guiding therapeutic development using the immune system.

3.2 KILLER CELLS

There are three classes of "killer" cells, each somewhat distinct and providing different approaches to killing pathogens. There are: (i) Cytotoxic T Cells (or Killer T cells or CTL), Natural Killer Cells (or NK cells) and (iii) Natural Killer T cells, NK-T cells. Each is different from the other and each has been used in a variety of ways in treating cancers[3]. I present some fundamental issues on each for coherency in our analysis. This is not a comprehensive overview but it focuses on the key points related to our overall argument

3.2.1 CTL of Killer T Cells

CTL or Killer T Cells have MHC-I molecules and CD-8 surface proteins. They can be activated through the adaptive immune system. Activation is via IL-2 increase via T Cell helpers. CTLs can bind to a target cell a d they then can conjugate which allows for granule exocytosis which kills the target and then also the CTL to progress to other targets. There are two pathways by which this attack can take; Fas pathway approach and the performin-ganzyme approach.

As Steer et al note:

Although anti-cancer immunity involves both the innate and adaptive immune systems, it is generally held that CD8þ cytotoxic T lymphocytes (CTL) are the most potent anti-tumour effector cell. The T-cell immune response can be broken down into the following steps, all of which need to be fulfilled for effective anti-tumour CTL to be generated:

[3] See Kindt et al, Kuby Immunology, Freeman (New York) 2007; pp 353-368.

(1) tumour antigen(s) must be present, and

(2) these must be presented in a context which is seen as dangerous by the immune system;

(3) antigens must be acquired and presented by antigen presenting cells (APC) in the draining lymph node;

(4) specific T cells must then recognize and respond to tumour antigen by proliferating, exiting the lymph node, recirculating and entering the tumour as CTL and

(5) once within the tumour they need to overcome the local immunosuppressive environment before they can kill tumour cells.

In addition, memory cells may need to be generated to produce a sustained response. It is clear that a growing tumour has managed to escape this process. Failure of the anti-tumour immune response can occur at one or more of these steps. Targeting rate limiting steps with therapies designed to boost the immune response can improve anti-tumour immunity.

In addition to specifically targeted immune therapies, it is also now clear that many traditional cancer therapies can improve key aspects of anti-cancer immunity by inducing tumour cell death in a way that is immunostimulatory or by modulating tumour induced immunosuppression.

3.2.2 NK Cells

NK cells are not normal T cells and they deviate from the T cell line earlier in the developmental process. They account for between 5% to 10% of the circulating lymphocytes. They work by producing cytokines and are generally considered a part of the innate immune system.

NK cells have both activation and inhibition receptors. They act in such a manner as to becoming active or inactive by a balancing of activation, it is a thresholding effect.

NK cells can be signaled by Interferons, TNF, IL-12 and IL-15. These have been used by researchers in attempts to activate the NK system.

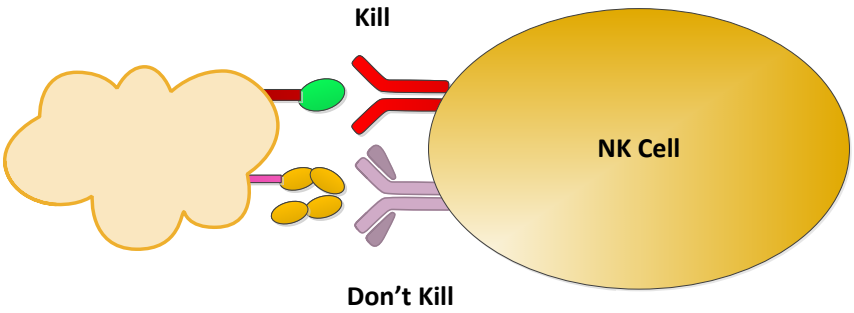
The receptors are lechtin like or immunoglobulin like. The lechtin receptors bind proteins and not lechtins. The second receptors bind HLA-B and HLA-C.

There are inhibitory receptors which are immunoglobulin like such as ILT/LIR as well as KIR, called killer inhibitory receptors.

NK cells have activating and inhibiting ligands. Thus an MHC-I represent a cell which is self and thus has an inhibitory reaction. A second receptor may reflect a viral infection and thus may activate. The actual activation is a balance between inhibition and activation. If the activation is strong enough then even though there may be a inhibitory self-recognition it may be overcome

by the activating ligand. This may be a pathway for cancer management. This mechanism is common and is a key point in the discussion of the set points focused on herein.

NK recognizes a marker on the surface and it decides to "kill" the cell. But it also recognizes the MHC I market as self and then does NOT Kill the cell. The MHC I acts as an inhibitor.



What should be noted is the general simplicity of this model. One must remember that there are a multiplicity of these receptors and not just one, that the receptors to function must deal with internal pathways and that there are a competing set of different receptors. The process is just not as simple as portrayed. This is why on the one hand the Chen and Mellman model has interest.

Now a more detailed discussion by Caligiuri notes:

Years ago, the histologic and functional definition of an NK cell was that of a large granular lymphocyte that could kill a target cell "naturally," that is, in a spontaneous fashion that did not require any priming and was not restricted by the target cell's expression of major histocompatibility complex (MHC) molecules. Experiments in mouse models of bone marrow graft rejection led to the proposal that NK cells would kill any target that lacked self–major histocompatibility complex (MHC) class I molecules (the "missing self" hypothesis).8 This extraordinary idea was developed before anyone knew what the NK cell was using to "see" its targets.

It is now clear that NK cells have a multitude of inhibitory and activating receptors that engage MHC class I molecules, MHC class I–like molecules, and molecules unrelated to MHC. Thus, NK cells are indeed restricted in what target cells they can engage by the expression of the target's MHC ligands, but in a very complex fashion that remains incompletely understood. Notably, orthologs of more recently discovered NK-cell receptor families cannot be found beyond mammals, suggesting that the composite modern day NK cell emerged well after T and B cells appeared to define the vertebrate adaptive immune system.

Furthermore, the complementary roles that NK and cytolytic T cells have in target recognition and host defense, and their similar mechanisms of cytolysis, suggest that these 2 cell types may have each evolved from a common ancestral cytolytic effector cell. Finally, a subset of human NK cells produce abundant cytokines with modest or no ability to lyse target cells. Thus, the

older idea of an NK cell as an ancestral forerunner or as a cell defined by a simple function no longer applies. The traditional cell surface phenotype defining human NK cells within the lymphocyte gate on the flow cytometric analyzer shows an absence of CD3 (thereby excluding T cells) and expression of CD56, the 140-kDa isoform of neural cell adhesion molecule (NCAM) found on NK cells and a minority of T cells.

He then goes on to describe what they do in some detail:

Thus, far it has been fully appreciated that NK cells can secrete cytokines and chemokines that influence the host's immune response, and/or kill certain infected or transformed cells via perforin/granzyme or death receptor (e.g., Fas, TRAIL)–related pathways.

Interferon gamma (IFN-) is considered the prototypic NK-cell cytokine, and its production by NK cells is known to shape the Th1 immune response, activate APCs to further up-regulate MHC class I expression, activate macrophage killing of obligate intracellular pathogens, and have antiproliferative effects on viral- and malignant-transformed cells. For many of these functions, it would make sense for NK cells to be in close proximity to APCs and T cells.

Indeed, the subset of NK cells that is the most potent producer of IFN- (i.e., CD56bright NK) is primarily located in the parafollicular T cell– and APC-rich region of SLT.21

As Pittari et al note:

The function of NK cells is governed by a set of germline- encoded activating or inhibitory receptors referred to as killer immunoglobulin-like receptors (KIRs). The extracellular domain determines which HLA class I molecule NK cells recognize, whereas the intracytoplasmic domain transmits either an activating or an inhibitory signal. KIRs are monomeric receptors with either 2 (KIR2D) or 3 (KIR3D) immunoglobulin-like domains, and are further subdivided into those with long (L) cytoplasmic tails (KIR2DL and KIR3DL) and short (S) cytoplasmic tails (KIR2DS and KIR3DS). Long-tail KIRs generate an inhibitory signal through the recruitment of the SH2-domain- containing tyrosine phosphatase 1 protein (SHP1). Short- tail KIRs possess truncated portions that transduce activating signals via tyrosine phosphatase of DAP12 and other proteins.

The NK receptors are also a key element for potential immunotherapy. The KIR receptors are especially the case.

As Vivier et al note:

NK cells were originally described as cytolytic effector lymphocytes, which, unlike cytotoxic T cells, can directly induce the death of tumor cells and virus-infected cells in the absence of specific immunization; hence their name.

Subsequently, NK cells have been recognized as major producers of cytokines such as interferon-g (IFN-g) in many physiological and pathological conditions.

NK cells also produce an array of other cytokines, both proinflammatory and immunosuppressive, such as tumor necrosis factor–a (TNF-a) and interleukin (IL)–10, respectively, and growth factors such as GM-CSF (granulocyte macrophage colony-stimulating factor), G-CSF (granulocyte colony stimulating factor), and IL-3. NK cells also secrete many chemokines, including CCL2 (MCP-1), CCL3 (MIP1-a), CCL4 (MIP1-b), CCL5 (RANTES), XCL1 (lymphotactin), and CXCL8 (IL-8).

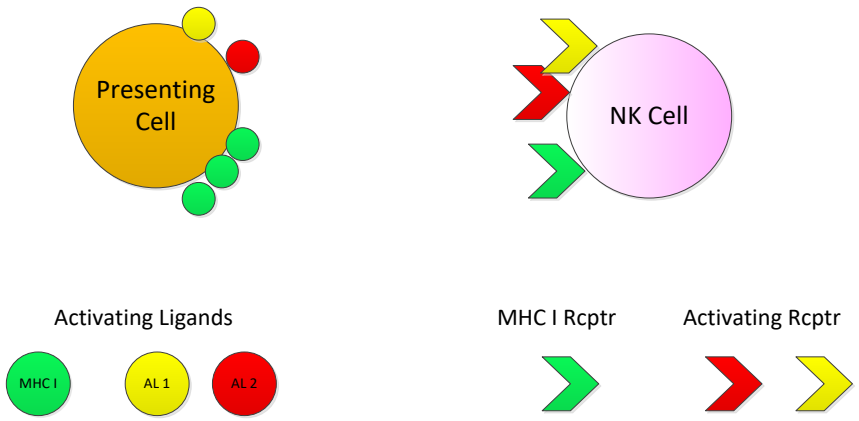
Whereas the biological function of the growth factors secreted by NK cells remains to be clarified, their secretion of chemokines is key to their colocalization with other hematopoietic cells such as dendritic cells (DC) in areas of inflammation. Furthermore, the production of IFN-g by NK cells helps to shape T cell responses in lymph nodes, possibly by a direct interaction between naïve T cells and NK cells migrating to secondary lymphoid compartments from inflamed peripheral tissues and by an indirect effect on DC.

NK cell–mediated killing of target cells also impacts T cell responses, possibly by decreasing the antigenic load and/or because target cell debris might promote antigen cross-presentation to CD8+ cytotoxic T cells. Although NK cells can positively or negatively influence host T and B cell immunity, depending on the nature of the antigenic challenge, the emerging notion is that NK cells are not only cytolytic effector cells against microbeinfected cells or tumor cells. Rather, NK cell– mediated cytotoxicity and cytokine production impact DC, macrophages, and neutrophils and endow NK cells with regulatory function affecting subsequent antigen-specific T and B cell responses.

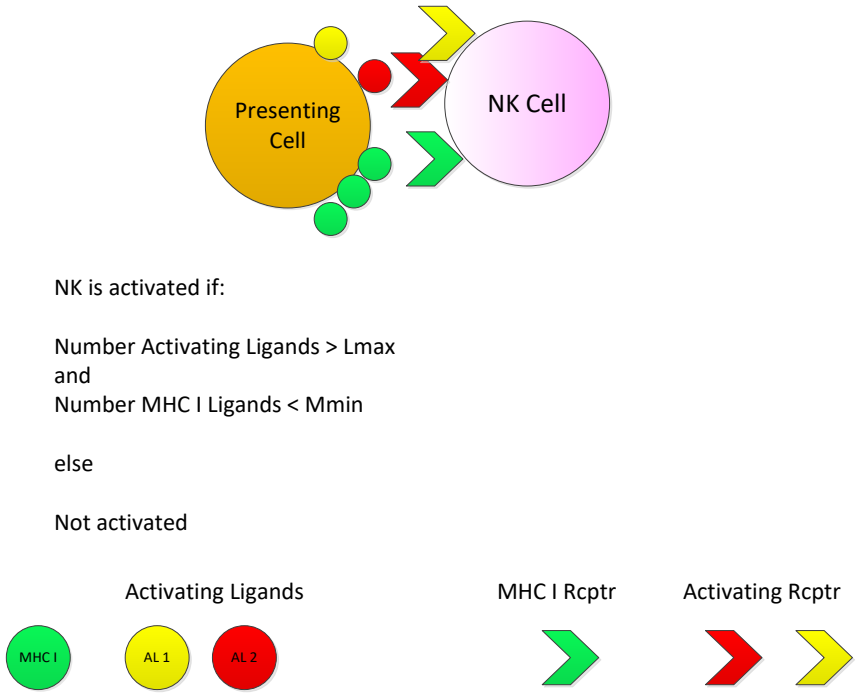
Conversely, the "natural" effector function of NK cells has been revisited. NK cells require priming by various factors, such as IL- 15 presented by DC or macrophages, IL-12 or IL-18 , to achieve their full effector potential, highlighting the intimate regulatory interactions between NK cells and other components of the immune response.

Thus, NK cells, like T and B cells, participate in the immunity in many different ways and undergo a process of functional maturation to fulfill these functions.

Now Vivier et al have described the rather interesting manner in which NK calls can be activated or inhibited which in a sense presages the work of Chen and Mellman. Simply, it is a bit of majority voting by ligands and receptors. We demonstrate this below. Activating ligands can attach to receptors as equally as inactivating.



Then below we demonstrate a somewhat simple majority voting scheme whereby the combination, subject to some putative weighting, can effect either activation or inactivation.



The problem with the above is that we really do not know the threshold. Furthermore we fundamentally do not understand the complexity of the decision making process inside one of these cells. Frankly that will be the challenge in pursuing this area.

Following Vivier et al we note:

NK cells are equipped with an array of receptors that can either stimulate NK cell reactivity (activating receptors) or dampen NK cell reactivity (inhibitory receptors). Activating receptors include receptors that interact with soluble ligands such as cytokines and receptors that interact with cell surface molecules.

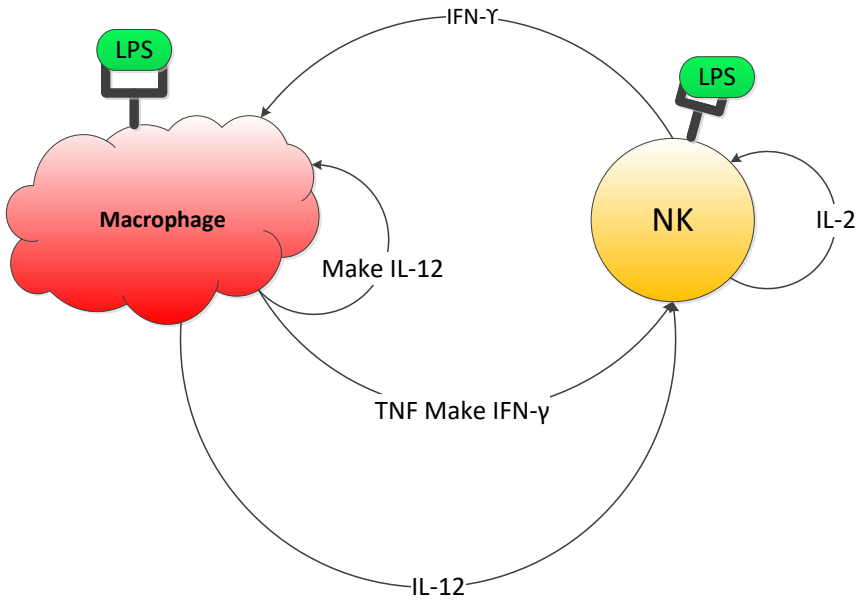
Cytokine receptors that are coupled to the common gamma chain (gc), such as IL-15R, IL-2R, and IL-21R, are involved in NK cell development and effector function. In particular, IL-15 is required for the maturation and survival of NK cells, consistent with the absence of circulating NK cells in SCIDX1 patients and in mice lacking IL-15 or IL-15R components. Cytokine receptors that are linked to the adapter protein MyD88 are also important for NK cell maturation, namely IL-1R in humans and IL-18R in the mouse.

NK cells exert their biological functions by various means. NK cells can kill a variety of target cells, including virus-infected cells and tumors, in the absence of antibody. In the case of viruses, the mouse Ly49H activating receptor recognizes a cytomegalovirus-encoded ligand (m157) (23, 24), and NKp46 has been reported to interact with hemagglutinins derived from influenza and parainfluenza viruses (25).

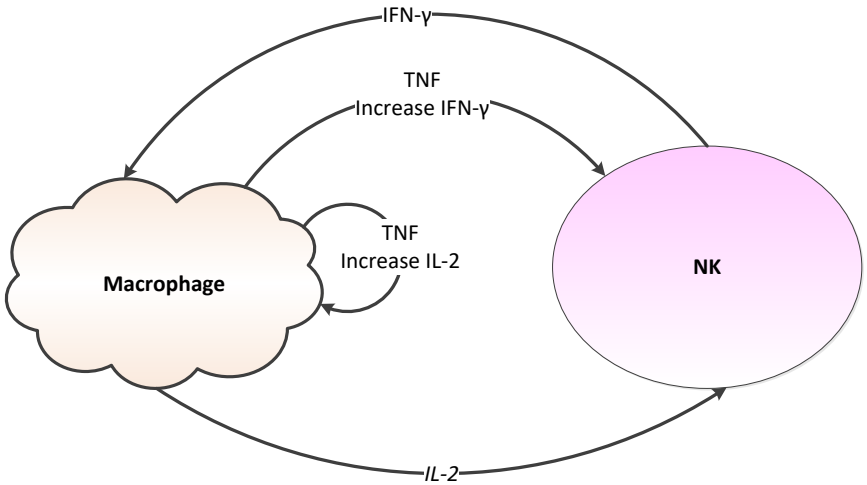
NK cells are also able to detect antibody-coated cells through the FcgRIIIA (CD16) cell surface receptor and to exert antibody-dependent cell cytotoxicity (ADCC) and cytokine production. CD16 is coupled to the CD3z and FcRg signal transduction polypeptides bearing intracytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs).

The natural cytotoxicity receptors (NKp46/NCR1, NKp44/NCR2, and NKp30/ NCR3) are also potent activation receptors linked to the ITAMbearing CD3z, FcRg, or DAP12 molecules. In mice, the NK1.1 (Nkrp1c) molecule on CD3– cells has been a useful marker for NK cells, but its expression is confined to only certain strains of mice. NKp46 appears to be the most specific NK cell marker across mammalian species, although discrete subsets of T cells also express it

Now shown below we depict the result of this activation process. There is a flow of Interferons further activating the NK and with the macrophage introduction of a pathogen identifier, in this case a lipo-poly saccharide, LPS, we see the NK then activated and beginning its response.



The figure below is another depiction of this process.

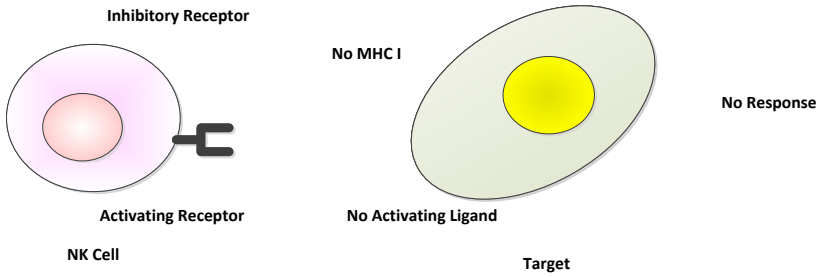


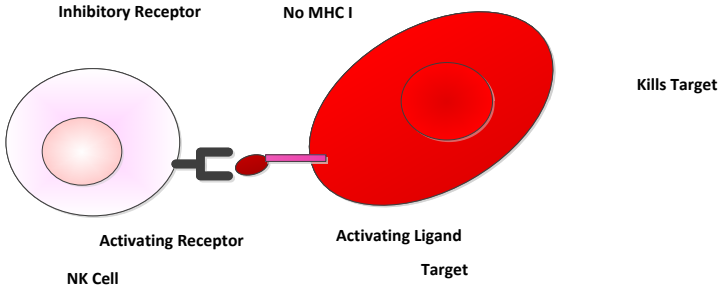
Vivier et al summarizes the various NK cell receptors by function.

Function/ Receptor	Activating Receptors	Inhibitory	Cytokine Receptors	Adhesion Receptors	Activating Adaptors
	NKp46	h KIR-L	IL-1R	CD2	CD3ζ, FcRγ
	CD16	h LILRB1	IL-2R	DNAM-1	CD3ζ, FcRγ
	h NKp30	CD94/NKG2A	IL-12R	β1 integrins	CD3ζ, FcRγ
	h NKp44	m Inh. Ly49	IL-15R	β2 integrins	DAP12
	h NKp80	m NKR-P1B	IL-18R		FcRγ
	m NKR-P1C	m NKR-P1D	IL-21R		DAP10
	NKG2D	KLRG-1	IFNAR		DAP12
	m NKG2D-S	TIGIT			DAP12
	h KIR-S	CEACAM-1			DAP12, DAP10
	m Act. Ly49	LAIR-1			DAP12
	CD94/NKG2C				SAP, EAT2
	CRACC				SAP
	Ly9				SAP, EAT2
	CD84				SAP
	NTBA				SAP, EAT2,
	2B4				ERT

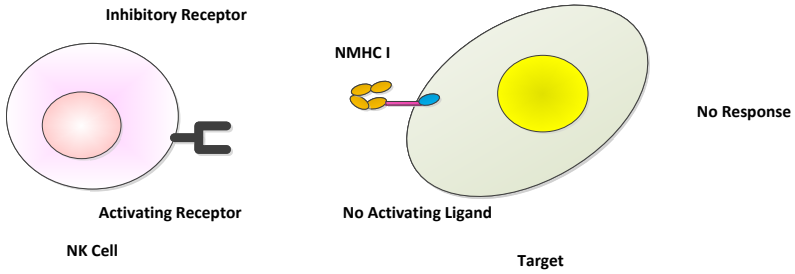
This is clearly a complex set of receptors which serve a multiplicity of functions. Vivier et al also discuss the question of NK being adaptive as well as innate. NK cells are quite powerful and have become cells of interest in a variety of cancer immunotherapeutic applications as we shall show latter.

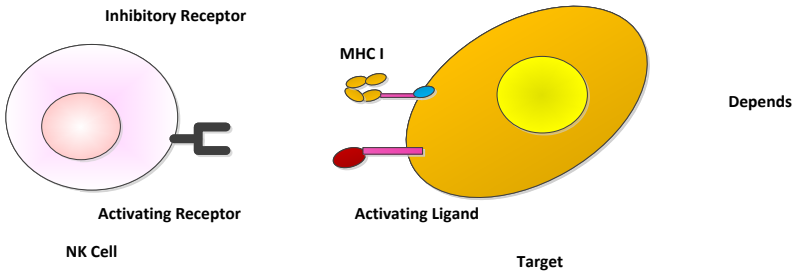
The following graphically demonstrate some of these options:





Then below are the other two options.





These four can be summarized in the Table below.

	Target Activating On	Target Activating OFF
Target Inhibitory On	Depends on Balance	NK Attacks
Target Inhibiting Off	No Response	No Response

Now in our current discussion this simplistic Table is for one Activator and One Inhibitor. The question surrounding the work of Chen and Mellman is:

How do we construct a model wherein the response is a complexity of:

1. Multiple Numbers of the same Inhibitors and/or activators

2. Multiple different Inhibitors and Activators

3. Multiplicity of pathway alterations resulting in variations of resulting responses.

3.2.3 NK T Cells

The NK T cell is neither a CTL nor an NK cell. It is a third variety somewhat in between. CTL are adaptive and NK are innate. The T cell receptor on NKT cells does not recognize MHC molecules and it has markers similar to both NK and CTL.

As Ibarrondo et al note:

Invariant natural killer T cells (Type I NKT cells or iNKT) are a subset of T cells that express a restricted repertoire of T-cell receptors (TCR); in humans the iNKT TCR alpha chain presents a Va24-JaQ rearrangement that preferentially pairs with a semiinvariant Vb11 b-chain. The iNKT TCR recognizes glycolipid antigens presented by CD1d, a major histocompatibility complexlike molecule present on the surface of antigen-presenting cells, and that is highly expressed by myeloid dendritic cells (mDCs). iNKT cells are actively recruited to infection sites, where they respond to cytokines and interact with CD1d + mDC. In response to stimuli, iNKT cells can release large amounts of regulatory cytokines and are believed to play a pivotal role in the determination of innate and adaptive immune system responses.

iNKT cells can be subdivided into three subsets: CD4 + , CD8 + and CD42/CD82 double negative (DN). The CD4 + subset has a Th0 profile, being able to produce Th2 and Th1 cytokines such as interleukin 4 (IL-4) and interferon gamma (IFN-c). DN iNKT cells produce large amounts of Th1 cytokines such as INF-c and tumor necrosis factor alpha (TNF-a), up-regulate perforin, and release low levels of Th2 cytokines in response to stimuli [7]. Finally, CD8 + iNKT cells constitute a Th1-only subse.

The balance of CD4 + versus DN and/or iNKT CD8 + iNKT cells is thought to be critical for proper modulation of immune responses to control inflammatory processes, auto-immunity, and immune surveillance of cancer. The pivotal role of iNKT cells in the regulation of the immune response makes them an attractive target for immunotherapy: the frequency and functionality of iNKT cells is frequently altered in patients with malignancies, autoimmune disorders, and viral infections. Blood iNKT cell frequencies fall in melanoma

As Stetson et al note:

Natural killer (NK) and NK T cells are tissue lymphocytes that secrete cytokines rapidly upon stimulation. Here, we show that these cells maintain distinct patterns of constitutive cytokine mRNAs.

Unlike conventional T cells, NK T cells activate interleukin (IL)-4 and interferon (IFN)- transcription during thymic development and populate the periphery with both cytokine loci previously modified by histone acetylation.

Similarly, NK cells transcribe and modify the IFN- gene, but not IL-4, during developmental maturation in the bone marrow. Lineage specific patterns of cytokine transcripts predate infection and suggest evolutionary selection for invariant but distinct types of effector responses among the earliest responding lymphocytes. NK cells are required for effective host defense against herpes viruses in mice and humans.

Although the precise evolutionary niche subserved by NK T cells is not completely clear, the capacity of NK T cells to activate rapid cytokine expression has been exploited to manipulate the outcomes of autoimmunity and cancer. Aside from their expression of common NK-associated surface antigens, such as NK1.1, NK T and NK cells share developmental requirements. Deficiencies in certain cytokines, such as IL-15 or lymphotoxin, or transcription factors such as Ets-1 or Irf-1, lead to loss of both cell lineages. Recent studies suggest their capacity to express cytokines rapidly may also be developmentally acquired .

Although other studies elegantly demonstrate how these cells become activated , the mechanisms underlying their rapid cytokine production or their distinct cytokine patterns, IFN- in the case of NK cells and both IL-4 and IFN- in the case of NK T cells, remain unknown. Elucidation of such mechanisms may have important implications for understanding polarized cytokine production by T cells in adaptive immune responses.

We demonstrate that NK T cells and NK cells, distinguished by their ability to mobilize effector cytokines rapidly after immunization or infection, reside in the periphery spontaneously poised with constitutive cytokine transcripts.

Modification of the respective cytokine loci in a manner promoting access by transcription factors correlates with the presence of cytokine mRNAs. Unlike conventional T cells, NK T and NK cells activate transcription of cytokine genes during early development in the thymus and bone marrow, respectively. In the case of IL-4 for NK T cells, neither the percentage of IL-4

3.3 SOME THEORY?

The previous brief summary lays out some of the issues inherent in the Chen and Mellman paper. To better understand let us now return to the ideas of Chen and Mellman. Specifically their definition of a "set point". They state:

The cancer–immune set point is the threshold that must be overcome to generate effective cancer immunity. The set point can be understood as a balance between the stimulatory factors (Fstim) minus the inhibitory factors (Finhib), which together must be equal to or greater than 1, over the summation of all T-cell antigen receptor (TCR) signals for tumour antigens. The cancer–immune set point is shown here:

$\int$ *(Fstim)* $-\int$ *(Finhib)* $\geq 1 / \Sigma\, n=1,\, y$ *(TCRaffinity* $\times$ *frequency)*

The set point is defined by the summation of the frequency of peptide–MHC–TCR interactions and TCR signalling in all anticancer CD8+ T-cell clones (mainly, the TCR affinity for the antigen–MHC class I complex) against antigens present in the cancer cells, including neoantigens and cancer-associated antigens, and the endogenous balance of the positive and negative immune regulators that are inherent to each host or patient.

Now just what this means is somewhat open for debate because it is written by a biologist not a physical scientist and definitely not an engineer. Permit me to attempt an interpretation. First let us try to be specific about a definition. Namely some definition of a variable which is measurable.

Let us try to first understand the F terms.

Fstim: This is a stimulatory factor. What is it? One could guess it is some cell with an MHC I presenting some antigen Ag to a T cell receptor TCR. Should we examine cell by cell? Should we look at every possible T cell, namely ones that say are CD 8 T cells, or how about other immune cells. Why not include NK cells as well? Should we look at stem cells only, do we know what they are? Do we then count these for every T cell, for a mass of T cells, for what?

Finhib: We know some of these we believe. There is PD-1 and CTLA-4. They can block the T cell from attacking. We also suspect that there are many others we have yet to find. So let us simplistically assume we can model with the two mentioned. But what are we measuring? Are we measuring a single cell, a collection of cells, the totality of all cells? Are we measuring all stimulatory factors or just a few? Are we measuring all inhibitory factors or just the ones we know? Are we weighting some differently than others or the same?

This if we have two single cells and it has say 50 T cell receptors and 45 PD-1 receptors, then we can have activated say 35 of the TCR and have activate say 22 of the PD-1. Now what happens? Is activation by each TCR the same and can a TCR being activated be inhibited by an activated PD-1 on a one to one basis?

$$\int_{S_0'}^{S_1} F_{Stim}(r,t)drdt - \int_{S_0'}^{S_1} F_{Inhibit}(r,t)drdt > \alpha$$

or

$$\sum_{n=1}^{N}\left[\int_{S_0'}^{S_1} F_{Stimulate}(n;r,t)drdt - \int_{S_0'}^{S_1} F_{Inhibit}(n;r,t)drdt\right] > \beta$$

The above still has no physical meaning. Now let us consider T Cell Affinity. As Nicholich et al state:

Affinity refers to the steady-state association constant between a monovalent receptor and its ligand, in this case a single T-cell receptor (TCR) and peptide–MHC (pMHC) complex. Structural avidity is the steady-state association constant between multiple cell-bound receptors and ligands and is determined by the direct binding affinities of multiple TCRs to their pMHC complexes. Functional avidity depends on the relative kinetics of signalling that translate into measurable biological functions such as proliferation, cytokine production or cytolytic function. APC, antigen-presenting cell.

Now as Hsieh et al note:

TCR affinity: The strength of interaction between the T cell receptor and a single peptide–MHC complex.

As an abstraction that may be fine but as something used in a measurement and equation it is highly deficient.

Now as Daniels et al note:

To estimate the TCR affinity of the ligands comprising the selection boundary, we measured tetramer binding; which correlates with monomeric TCR–pMHC affinities, is performed on live cells and involves the participation of CD8. The binding characteristics of tetramers were determined on pre-selection OT-I double positive thymocytes at 37 uC. The dissociation constant (Kd) was calculated by nonlinear regression analysis and confirmed by homologous competition experiments.

The tetramer binding curves for Q4R7 (weakest negative selector), T4 (border ligand) and Q4H7 (strongest positive selector) overlapped. Their Kd values (Q4R7, 4869.5 nM; T4, 55610.1 nM; Q4H7, 5169.1 nM; n57, P50.455) and their half-lives (t1/2) were not significantly different (Table 1). However, heterologous competition assays showed that Q4R7 was more efficient than Q4H7 at inhibiting the binding of OVA tetramers.

or perhaps they mean something akin to this:

$$\sum_{n=1}^{N}\left[\int_{S_0'}^{S_1} F_{Stimulate}(n;r,t)drdt - \int_{S_0'}^{S_1} F_{Inhibit}(n;r,t)drdt\right] > \beta$$

or

$$\frac{\sum_{n=1}^{N}\left[\int_{S_0'}^{S_1} F_{Stimulate}(n;r,t)drdt - \int_{S_0'}^{S_1} F_{Inhibit}(n;r,t)drdt\right]}{\sum_{m=1}^{M} TCR_{Affinity}(m)\, f(m)} > \lambda$$

Now we know that there is a threshold effect for activating and suppressing. Namely there has to be more activators than suppressors. Just what that balance is of course is uncertain. Again, the statement has no physical meaning.

They continue:

This can be further influenced by other elements of immunity, including tumour-derived immunomodulatory components, as well as by exogenous factors such as infection and exposure to pharmacological agents. A given patient with cancer may have a low set point, making it easier to generate an anticancer immune response, or a high set point, which makes it more difficult.

The aim of immunotherapy is to increase Fstim, decrease Finhib or increase TCR signalling to drive progression of the cancer-immunity cycle. These values are difficult to quantify with current techniques but represent a useful theoretical construct. It is probable that the cancer–immune set point of a particular person is already determined by the time of clinical presentation, driven by the inherent immunogenicity of the tumour and by the responsiveness of the individual's immune system.

Although it is reasonable to assume that various lines of cancer therapy or changes in environmental factors might alter Fstim and Finhib, such changes might only be transient. Often, the set point that is identified using pretreatment biopsies is similar to the set point determined by biomarker profiling from biopsies taken on progression after therapy.

Likewise, despite the continued accumulation of mutations in a tumour as a function of time, primary and metastatic lesions can exhibit similar immune profiles. The features that determine the set point may therefore reflect genetic factors that are specific to a given tumour, the genetics of the person with cancer, or the extent to which antitumor immunity had developed initially. Conceivably, immunotherapy may work as a consequence of either its direct effect on Fstim and Finhib (that is, by assisting the completion of a single revolution of the cancer-immunity cycle) or its ability to alter the set point (for example, by propagating the cancer-immunity cycle, which enhances the cancer-specific T-cell response).

Although largely conceptual, the idea of a set point provides a framework to help organize the torrent of clinical and biomarker data that will emerge over the coming months and years. The number of targets that could prove effective for cancer immunotherapy is great; the number of potential combinations of therapeutic agents that are directed against these targets (or combinations of such agents with conventional standard-of-care agents) is even greater.

Thus, let us try and construct meaning which may be measurable and verifiable as well as actionable. Consider the following model:

1. Let us assume we have a tumor cell. Let us assume there are N possible activator ligands and M inhibitor ligands.

2. Let us assume that for each of the above ligands we have on a T cell some receptor. If there is a ligand without a receptor we shall ignore it.

3. Assume we can count and differentiate the differing ligand-receptor possibilities on a cell.

4. Now calculate the following:

$$\frac{\sum_{k=1}^{K}\alpha_k N_{activator,k}-\sum_{j=1}^{J}\beta_j N_{Inhibitor,j}}{\sum_{k=1}^{K}N_{activator,k}+\sum_{j=1}^{J}N_{Inhibitor,j}}\leq\lambda$$

Consider the following model:

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4. Now calculate the following:

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However, if we have to consider the binding dynamics we would have:

$$TCR+pepMHC \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} TCR:pepMHC$$

where TCR is the T Cell receptor and pepMHC is, the antigen presenting particle on the MHC surface molecule complex.

Thus, N as above is a random variable, in fact a random process. That is for any activator or inhibitor at any time:

$$N(t) = \sum_{k=1}^{K} n_k(t)$$

where

$$n_k(t) = \begin{cases} 1; P[n=1] = p \\ 0; P[n=0] = 1 - p = q \end{cases}$$

and

$$E[N(t)] = pK$$

That is each N(t) is a random process where it is characterized by two parameters; the maximum number and the probability of binding. If K were large enough then we could use the central limit theorem to provide a Gaussian distribution with mean and variance. Namely:

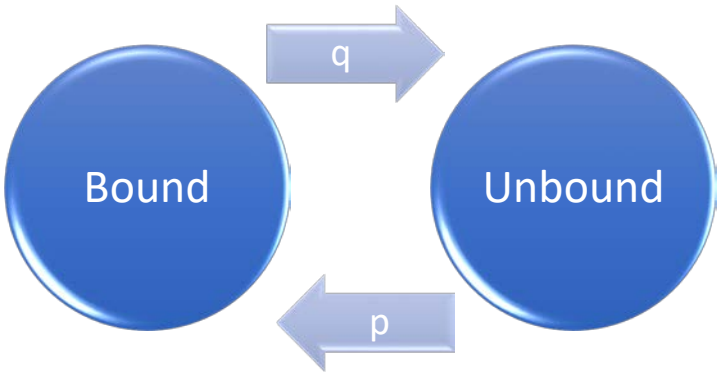
$$p_{n_i}(x) = N(p_i K_i, p_i q_i K_i)$$

where N represent the normal density with mean pK and variance pqK.

Thus, when we examine the statistic above we can write it as:

$$\Lambda(t) = \frac{\Delta(t)}{\Pi(t)} \geq \lambda$$

where numerator and denominator are Gaussian. However, if the number or receptors is large then both numerator and denominator reduce to near constants. On the other hand, if there is but one pair of each we have a random process.



This process is commonly known as the "Telegraph Process"[4]. Namely it is a simple on-off system and as such it would either suppress or activate the process. Depending on how large the inhibition is, or the activation is, will be reflected in the time the cells are controlled by the immune system. From a therapeutic perspective, the question is; is there a mechanism to keep it active at a higher rate?

[4] See Parzen, Stochastic Processes, Holden Day, 1962.

Namely, we count the number of different activators and the number of different inhibitors and then weight them by some metric, yet to be determined, and then weigh them by the total present.

This approach may have merit. The weights may be unity, but that is a mere guess. The weights may be reflective of the enzymatic consistency of the contact. Frankly we just do not know but it is worth exploring.

3.4 T CELL MECHANICS

We briefly will examine the now classic model of Check Points, specifically the PD-1 Check Point which has received a great deal of attention.

From Freeman, we have an excellent summary description:

T cell activation requires a TCR mediated signal, but the strength, course, and duration are directed by costimulatory molecules and cytokines from the antigen-presenting cell (APC).

An unexpected finding was that some molecular pairs attenuate the strength of the TCR signal, a process termed coinhibition.

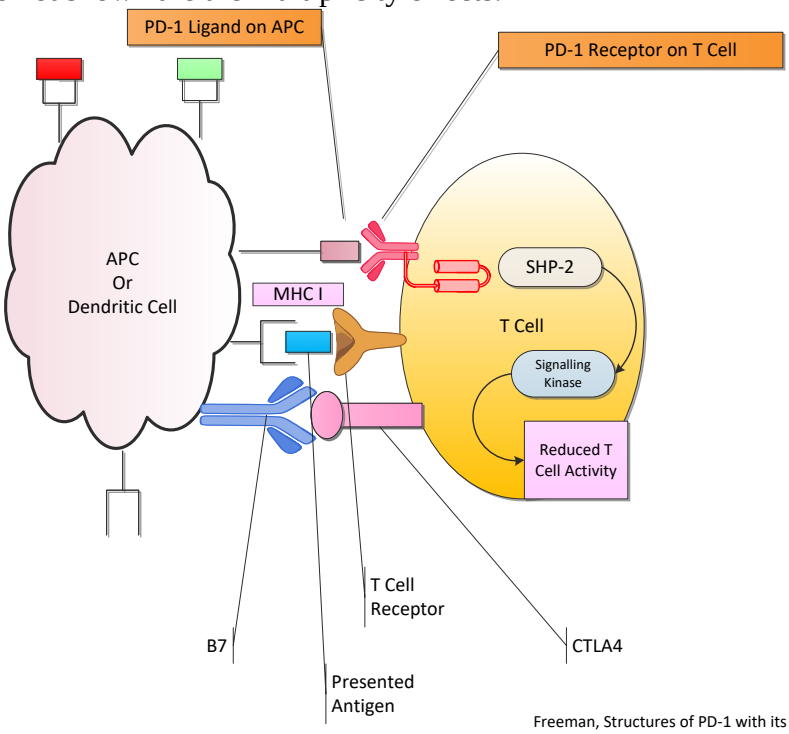
The threshold for the initiation of an immune response is set very high, with a requirement for both antigen recognition and costimulatory signals from innate immune recognition of ‘‘danger’’ signals. Paradoxically, T cell activation also induces expression of coinhibitory receptors such as programmed death-1 (PD-1).

Cytokines produced after T cell activation such as INF- and IL-4 up-regulate PD-1 ligands, establishing a feedback loop that attenuates immune responses and limits the extent of immune-mediated tissue damage unless overridden by strong costimulatory signals. PD-1 is a CD28 family member expressed on activated T cells, B cells, and myeloid cells. In proximity to the TCR signaling complex, PD-1 delivers a coinhibitory signal upon binding to either of its two ligands, PD-L1 or PD-L2.

Engagement of ligand results in tyrosine phosphorylation of the PD-1 cytoplasmic domain and recruitment of phosphatases, particularly SHP2. This results in dephosphorylation of TCR proximal signaling molecules including ZAP70, PKC, and CD3, leading to attenuation of the TCR/CD28 signal.

The role of the PD-1 pathway in peripheral T cell tolerance and its role in immune evasion by tumors and chronic infections make the PD-1 pathway a promising therapeutic target. Two recent papers have determined the structures of the PD-1/PD-L1 and PD-1/PD-L2 complexes. PD-L2 (B7-DC; CD273) is inducibly expressed on dendritic cells and macrophages, whereas PD-L1 (B7-H1; CD274) is broadly expressed on both professional and nonprofessional APCs as well as a wide variety of nonhematopoietic cell types. The PD-1 pathway is important for the maintenance of peripheral T cell tolerance.

This process is shown graphically below. All three elements are shown; activator, inhibitor, and pathway. What is not shown are the multiplicity effects.



Freeman, Structures of PD-1 with its ligands: Sideways and dancing cheek to cheek

Now if we have a simple model as above we then consider for a therapeutic a mechanism for blocking the Check Point. Namely design for example a monoclonal antibody, Mab, which can overpower the PD-1 receptor and inhibit is reaction. This has been the basis for many such therapies.

rom Galluzzi et al we have the following list of Mabs used or in study for various cancers.

Alemtuzumab	**Chronic lympocytic leukemia**	**2001**	**Selective recognition/opsonization of CD52+ neoplastic cells**
Bevacizumab	Colorectal carcinoma Glioblastoma multiforme Cervical carcinoma Lung carcinoma Renal cell carcinoma	2004	VEGFA neutralization
Brentuximab vedotin	Anaplastic large cell lymphoma Hodgkin's lymphoma	2011	Selective delivery of MMAE to CD30+ neoplastic cells
Blinatumumab	Acute lymphoblastic leukemia	2014	CD3- and CD19-specific BiTE
Catumaxomab	Malignant ascites in patients with EPCAM+ cancer	2009	CD3- and EPCAM-specific BiTE
Ipilimumab	Melanoma	2011	Blockage of CTLA4-dependent immunological checkpoints
Nivolumab	Melanoma	2014	Blockage of PDCD1-dependent immunological checkpoints
Pembrolizumab	Melanoma	2014	Blockage of PDCD1-dependent immunological checkpoints
Cetuximab	Head and neck cancer Colorectal carcinoma	2004	Inhibition of EGFR signaling
Denosumab	Breast carcinoma Prostate carcinoma Bone giant cell tumors	2011	Inhibition of RANKL signaling
Gemtuzumab ozogamicin	Acute myeloid leukemia	2000	Selective delivery of calicheamicin to CD33+ neoplastic cells
Ibritumomab tiuxetan	Non-Hodgkin lymphoma	2002	Selective delivery of 90Y or 111In to CD20+ neoplastic cells
Panitumumab	Colorectal carcinoma	2006	Inhibition of EGFR signaling
Pertuzumab	Breast carcinoma	2012	Inhibition of HER2 signaling
Obinutuzumab	Chronic lymphocytic leukemia	2013	Selective recognition/opsonization of CD20+ neoplastic cells
Ofatumumab	Chronic lymphocytic leukemia	2009	Selective recognition/opsonization of CD20+ neoplastic cells
Ramucirumab	Gastric or gastroesophageal junction adenocarcinoma	2014	Inhibition of KDR signaling
Rituximab	Chronic lymphocytic leukemia Non-Hodgkin lymphoma	1997	Selective recognition/opsonization of CD20+ neoplastic cells
Siltuximab	Multicentric Castleman’s disease	2014	IL-6 neutralization
Tositumomab	Non-Hodgkin lymphoma	2003	Selective recognition/opsonization of, or selective delivery of 90Y or 111In to, CD20+ neoplastic cells
Trastuzumab	Breast carcinoma Gastric or gastroesophageal junction adenocarcinoma	1998	Selective recognition/opsonization of, or selective delivery of mertansine to, HER2+ cancer cells

The interesting observation regarding Mabs is that they require some check point type inhibitor plus they must not cause massive check point failures elsewhere. One should always be concerned with what can be called the "carpet bombing" effect. Namely in targeting one aberrant cell we manage to kill an excessive number of bystanders to the detriment of the patient.

3.5 Back to T Cell Immune Set Points

We now return to following Chen and Mellman and their observations. They note:

The role of the immune system in cancer remained unappreciated for many decades because tumors effectively suppress immune responses by activating negative regulatory pathways (also called checkpoints) that are associated with immune homeostasis or by adopting features that enable them to actively escape detection.

Two such checkpoints, cytotoxic T-lymphocyte protein 4 (CTLA4) and programmed cell death protein 1 (PD-1), have garnered the most attention so far.

CTLA4 is a negative regulator of T cells that acts to control T-cell activation by competing with the co-stimulatory molecule CD28 for binding to shared ligands CD80 (also known as B7.1) and CD86 (also known as B7.2).

The cell-surface receptor PD-1 is expressed by T cells on activation during priming or expansion and binds to one of two ligands, PD-L1 and PD-L2. Many types of cells can express PD-L1, including tumour cells and immune cells after exposure to cytokines such as interferon (IFN)-γ; however, PD-L2 is expressed mainly on dendritic cells in normal tissues. Binding of PD-L1 or PD-L2 to PD-1 generates an inhibitory signal that attenuates the activity of T cells. The 'exhaustion' of effector T cells was identified through studies of chronic viral infection in mice in which the PD-L1/PD-1 axis was found to be an important negative feedback loop that ensures immune homeostasis; it is also an important axis for restricting tumour immunity.

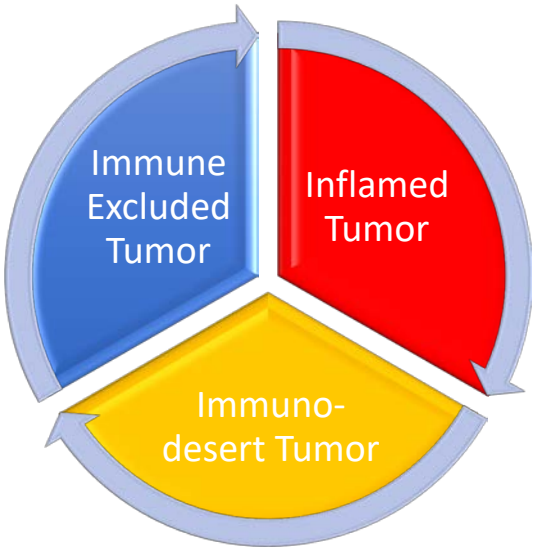
They then proceed to characterize three differing states of tumors with respect to their T cell response. They are:

1. Inflamed Tumor: This a tumor with lots of cells and penetrating the tumor space.

2. Immune Desert Tumor: This is a tumor with lots of cells but no significant penetration of the tumor space.

3. Immune Excluded Tumor: This is a tumor with a paucity of any T cells present.

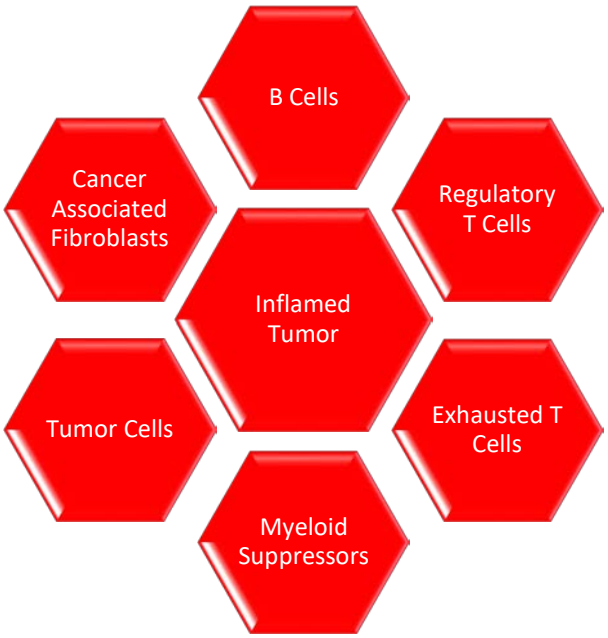
We depict those three types below.



Now we consider the descriptions as presented by Chen and Mellman:

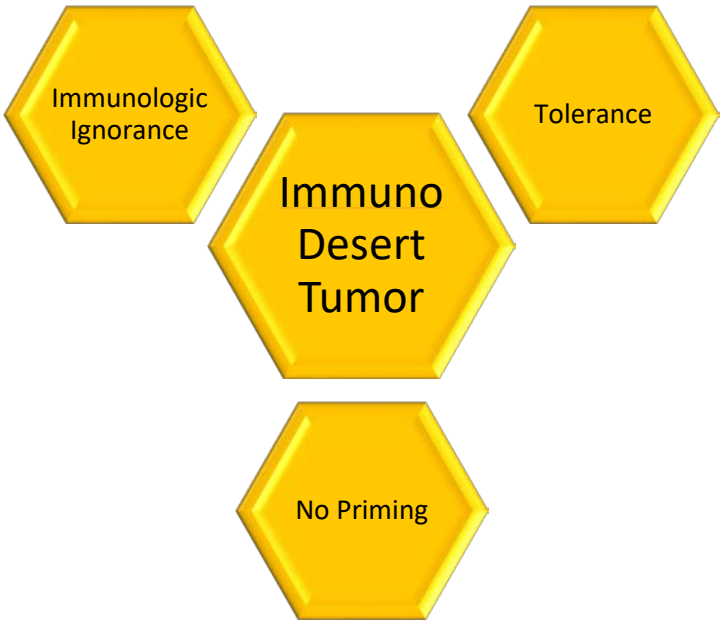
The first profile, the immune-inflamed phenotype, is characterized by the presence in the tumour parenchyma of both CD4- and CD8-expressing T cells, often accompanied by myeloid cells and monocytic cells; the immune cells are positioned in proximity to the tumour cells. Samples from inflamed tumors may exhibit staining for PD-L1 on infiltrating immune cells and, in some cases, tumour cells. Many proinflammatory and effector cytokines can also be detected by mRNA analysis in these sections of tumors. This profile suggests the presence of a pre-existing antitumor immune response that was arrested probably by immunosuppression in the tumour bed. Indeed, clinical responses to anti-PD-L1/PD-1 therapy occur most often in patients with inflamed tumors…

We depict the characteristics graphically below:



The second profile is the immune-excluded phenotype, which is also characterized by the presence of abundant immune cells. However, the immune cells do not penetrate the parenchyma of these tumors but instead are retained in the stroma that surrounds nests of tumour cells. The stroma may be limited to the tumour capsule or might penetrate the tumour itself, making it seem that the immune cells are actually inside the tumour. After treatment with anti-PD-L1/PD-1 agents, stroma-associated T cells can show evidence of activation and proliferation but not infiltration, and clinical responses are uncommon. These features suggest that a pre-existing antitumor response might have been present but was rendered ineffective by a block in tumour penetration through the stroma or by the retention of immune cells in the stroma. T-cell migration through the tumour stroma is therefore the rate-limiting step in the cancer–immunity cycle for this phenotype.

We depict the characteristics of this class below:



Finally, the third type is characterized as follows:

The third profile, the immune-desert phenotype, is characterized by a paucity of T cells in either the parenchyma or the stroma of the tumour. Although myeloid cells may be present, the general feature of this profile is the presence of a non-inflamed tumour microenvironment with few or no CD8-carrying T cells. Unsurprisingly, such tumors rarely respond to anti-PD-L1/PD-1 therapy. This phenotype probably reflects the absence of pre-existing antitumor immunity, which suggests that the generation of tumour-specific T cells is the rate-limiting step. The immune-desert phenotype and the immune-excluded phenotype can both be considered as non-inflamed tumors.

Thus, this does pose the question; how does one identify these cells and how could one move one category to the other for better response? Frankly one asks just what is happening from one class to another?

3.6 OBSERVATIONS

Set Points, Check Points, and other elements of the control of the immune system as a mechanism to understand and deal with cancer has been evolving at a rapid pace. Where the Check Point field seeks new and effective ligand-receptor pairs, the Set Point field seems to examine the process in a more holistic manner. Perhaps that is an approach which would enable a more systematic approach.

3.6.1 Cell Maturation and Differentiation

How does this process change as a cell matures? What of cell differentiation. T cells like many of the lymphoid line go through varying degrees of maturation. Thus, we ask: what is the difference?

3.6.2 Stem Cells

We have discussed the stem cell constructs at length. In McGarty (Stem Cells) we have tried to bring some of these ideas up to data. The problem is that stem cells may very well have different markers than the cells we can attack with the tools at hand. Thus, attacking PD-1 and CTLA4 markers may work for the mass of the tumor and result in shrinkage but it may totally miss the stem cell. How best to address this is uncertain?

3.6.3 Therapeutic Dimensions

What are the therapeutic dimensions of this principle? We have discussed a few here but there are many which present themselves.

3.6.4 CAR T Cells

CAR-T cells are "engineered" T cells which are designed by use of such tools as a lentivirus to attack a specific malignant cell. They have been shown to be useful for hematological cancers and have been examined for solid tumors. As Ramachandran notes in his Thesis:

As the name suggests, a CAR is a chimera of domains from different proteins assembled together to create a functional receptor. These novel receptors initiate a functional downstream effector T-cell signaling pathway when they encounter target antigen, usually the TAA on a cancer cell. This gives the opportunity to engineer a large variety of TAA-specific receptors targeting a broad range of cancer types.

CARs typically contain four domains

(a) extracellular antigen binding domain: It confers the antigen-specificity to the engineered T-cell. A majority of the engineered CARs for cancer therapy have antibody-derived antigen binding domains called single-chain variable fragment (scFv). CARs containing a scFv extracellular domain retain the specificity of an antibody. A major advantage of having scFv extracellular domain is that it bypasses the need for antigen presentation by MHC-I on tumor cells, as antibodies directly bind to cell surface antigens.

(b) Spacer or hinge region: It gives flexibility and length to allow proper dimerization of scFv, thus improving its stability. The most commonly used spacer regions are derived from IgG Fc CH2-CH3 domains, CD28 hinge domain and CD8α spacer domain

(c) Transmembrane domain: It determines the stability of CAR expression on cell surface. The most commonly used transmembrane regions are derived from CD3ζ CD4, CD8 and CD28 molecules138 and

(d) Cytoplasmic signaling domain(s): This region has the domains that provide the necessary downstream signaling for T-cell effector functions.

CARs are classified into different generations based on the number of cytoplasmic signaling domains namely first, second and third generation CARs. First generation CARs have only one cytoplasmic domain, usually T-cell activation signaling domain (CD3ζ chain). In addition to the T-cell activation domain second generation CARs have one extra co-stimulatory signaling domain, e.g., CD28, 4-1BB, ICOS or OX40 and third generation CARs have two extra co-stimulatory domains…

In a recent Technical Note McGarty has further developed the CAR-T cell concepts for both hematological and solid tumors. CAR-T are engineered to specific targets. The question then is; can a better understanding of set points allow for improved targeting for CAR-T cells or are CAR-T cells perforce of their design not really useful for attacking solid tumors?

3.6.5 Dynamic Models

The enzyme kinetics of the reactions on the surfaces of T cells and APC or tumor cells are critical. We have almost always assumed that once a protein is bound it stays. Yet we know it is not the case. Furthermore, when understanding the set point model, if we have a paucity of activators on a T cell it will not function. If the paucity is due to enzymatic action, then perhaps we can indirectly address the low level by increasing the retention via enzyme kinetic improvement.

3.6.6 Pathway Factors

The pathway factors are both integral to immunotherapeutic approaches, they facilitate the process inside a T cell for example, but they may also be poorly understood. Let us briefly review that issue. We must look at pathways from the perspective of the T cell and the tumor Ag presenting cell.

1. From the T cell perspective we have internal genetic pathways which facilitate the process of cytokine release. If there are faults on the pathway, then we would not expect the T cell to function. Thus, we may ask if these are somatic defects or a result of some change in the T cell.

2. From the perspective of the tumor cell, we know its pathways have usually been altered. Then does this altering result in the excess expression of inhibitors or the suppression of activators. Do the pathways alter the MHC I presentation efforts?

Both dimensions are worth examining.

3.6.7 Political Factors

A recent National Academies Report by Balogh et al present several policy issues regarding immunotherapy. The report was meant to present a simplified overview of immunotherapeutics as well as present some key policy issues. Concerns regarding costs, patient value, physician-patient expectations were discussed. Also was a discussion on evidence based approaches. The problem is that the experience is limited and the costs high. Furthermore what seemed not discussed was the fact that the complexity of this field is great and the depth of understanding by

physicians quite limited. One could say that most Oncologists are trained to administer chemotherapy, and have a limited if not aged understanding of the immune system.

4 STEM CELLS

Cancer Stem Cells ("CSC") and the related cells of origin, and similar cells have been examined in details by many researchers. Simply viewed, a stem cell is a particular cancer cell, whose presence via a plethora of means, it enables the growth and proliferation of the remaining body of a cancer. Removing a cancer stem cell, in principle, removes from the cancer the ability to grow and proliferate. This concept is important for the study of immunotherapy since it reflects an understanding of what cells to target. As relates to immunotherapy, one therefore may target all the other cancer cells but by leaving an active stem cell one allows for a rejuvenation of the malignancy. Our focus here is on prostate cancer since there is somewhat of an intellectual as well as lab based controversy worth examining.

Prostate Cancer, PCa, provides a unique target for examining these cells and at the same time has provided a fertile ground for disputes. Thus there is a strong disagreement as to whether the basal or luminal cell is the cell of origin and therein the CSC issue also arises. In a recent paper from the Tang Lab at MD Anderson they have present conclusions supporting a basal origin. In contrast Shen at Columbia has focused on luminal cells. In this note we attempt to bring an update to what we wrote in 2012 and provide some basis for comparing the various claims[5]. Fundamentally, we take no position in this debate[6].

Zhang et al state:

The prostate gland mainly contains basal and luminal cells constructed as a pseudostratified epithelium. Annotation of prostate epithelial transcriptomes provides a foundation for discoveries that can impact disease understanding and treatment. Here we describe a genome-wide transcriptome analysis of human benign prostatic basal and luminal epithelial populations using deep RNA sequencing. Through molecular and biological characterizations, we show that the differential gene-expression profiles account for their distinct functional properties. Strikingly, basal cells preferentially express gene categories associated with stem cells, neurogenesis and ribosomal RNA (rRNA) biogenesis.

Consistent with this profile, basal cells functionally exhibit intrinsic stem-like and neurogenic properties with enhanced rRNA transcription activity. Of clinical relevance, the basal cell gene-expression profile is enriched in advanced, anaplastic, castration-resistant and metastatic prostate cancers. Therefore, we link the cell-type-specific gene signatures to aggressive subtypes of prostate cancer and identify gene signatures associated with adverse clinical features.

[5] See Telmarc White Paper 85, Stem Cells, http://www.telmarc.com/Documents/White%20Papers/85%20Prostate%20Stem%20Cells.pdf and https://www.researchgate.net/publication/301222986_Prostate_Cancer_Stem_Cells?ev=prf_pub

[6] The reader is referenced to the White Papers referenced in this documents for details on specific topics. Also see Prostate Cancer: A Systems Approach by the author.

This is an argument for the basal cell being the origin of the CSC. They continue:

The current study has made the following significant findings.

First, our study uncovers unique SC- and EMT-enriched gene-expression profile in unperturbed basal cells that support the long-held hypothesis that the human prostate basal cell layer harbors primitive SCs.

Second, we report the surprising finding that basal cells are enriched in genes normally associated with neurogenesis. In contrast, luminal cells preferentially express proneural genes involved in neural signal response and processing. Consistently, primary basal cells can spontaneously or be induced to undergo 'neural' development in vitro, generating NSC-like cells. Combined with the SC features, these transcriptional programs provide a molecular understanding for the reported basal cell plasticity.

Third, basal cells express high levels of Pol I-associated rRNA biogenesis genes regulated, at least in part, by the MYC transcriptional programme. MYC is often found overexpressed in PCa, especially metastatic PCa. Increased transcription of rRNA genes by Pol I is a common feature of human cancer. Thus, our data may suggest a rationale for treating anaplastic PCa and CRPC with Pol I inhibition, as well as targeting MYC and the MYC-mediated transcriptional programme as a therapy for PCa.

Fourth, our deep RNA-Seq data provide a rich resource for epithelial lineage specific genes and markers in the human prostate.

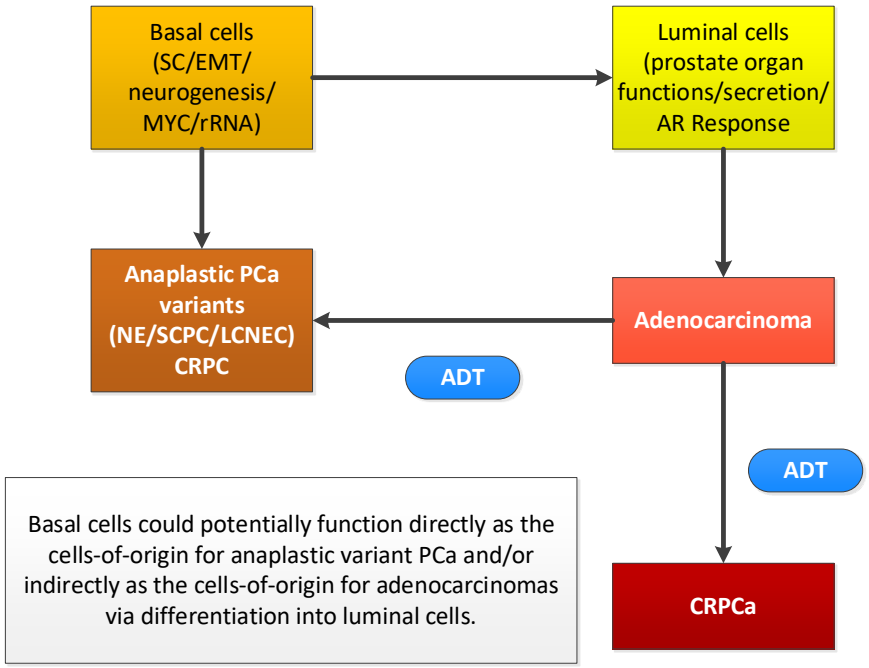
Fifth, distinct transcriptomes in basal and luminal cells also suggest cross communications between the two epithelial cell types, as well as between the epithelial compartment and the underlying stroma. Understanding such crosstalk will be instrumental for understanding the normal development and tumorigenesis of prostate. Although many of the signaling pathways mentioned in this study are poorly investigated in normal prostate epithelial biology, their functional involvement in PCa development and progression has been widely documented.

Last, the basal cell gene-expression profile is linked to adverse clinical features of PCa, indicating a 'biomarker' value of basal cell gene signature for aggressive PCa. Importantly, the molecular resemblance of basal cells to anaplastic PCa and CRPC provides a common molecular understanding of these diverse and poorly characterized aggressive PCa subtypes and implicates basal cells as the cell-of-origin for these variant PCa.

We present the summary of the Tang Lab model. The driver is a basal cell and the luminal cells seem to act if and only if driven by a basal cell process. Furthermore, the neuroendocrine case is shown as a direct and indirect result of the basal driver. We have recently discussed the neuroendocrine prostate cancers in our discussions of pro-NPY[7].

The Figure below is modified from the Wang et al paper and summarizes their concept.

[7] See https://www.researchgate.net/publication/292978295_pro-NPY_PCa_and_Neuroendocrine_Tumors



The above indicates the origin is from basal and then to luminal or the neuroendocrine cells, the latter being substantially less common.

Now the CSC and CCO debate, especially that related to PCa can be viewed in almost classical terms. In the 14th century as Medical Schools at Montpellier, Bologna, Paris and Oxford studied Galen and other then classic medical texts, the use of logic was compelling and demanded. The Trivium, Grammar, Logic and Rhetoric, was required of any student studying the field. This was because studying Galen demanded logic. Processes that were diagnostic or prognostic demanded logical consistency more than phenomenological verification. Strangely in the case of the CSC perhaps logical consistency is pari passu with that phenomenon. One of the major problems is defining the terms in such a manner that they can be consistently phenomenologically compared.

4.1 Definitions

As we have indicated, one of the more difficult issues when discussing CSCs is the definition. Phenomenological observations have been reduced to definitions and in turn the definitions have been used to search for CSCs. This can be a bit circular at times and may very well be one of the sources of confusion. Let us begin with the paper by Jordan et al from a decade ago in NEJM:

Many studies performed over the past 30 to 40 years, when viewed collectively, have shown that the characteristics of stem-cell systems, the specific stem-cell properties described above, or both, are relevant to some forms of human cancer. Biologically distinct and relatively rare populations of "tumor-initiating" cells have been identified in cancers of the hematopoietic system, brain, and breast.

Cells of this type have the capacity for self-renewal, the potential to develop into any cell in the overall tumor population, and the proliferative ability to drive continued expansion of the population of malignant cells. Accordingly, the properties of tumor-initiating cells closely parallel the three features that define normal stem cells. Malignant cells with these functional properties have been termed "cancer stem cells".

This frankly is a cumbersome definition. They describe stem cells; a necessary part of the definition as follows:

Stem cells occur in many different somatic tissues and are important participants in their physiology. Populations of cells that derive from stem cells are organized in a hierarchical fashion, with the stem cell residing at the apex of the developmental pathway. Stem cells have three distinctive properties: self renewal (i.e., at cell division, one or both daughter cells retain the same biologic properties as the parent cell), the capability to develop into multiple lineages, and the potential to proliferate extensively. The combination of these three properties makes stem cells unique. The attribute of self-renewal is especially notable, because its subversion is highly relevant to oncogenesis and malignancy. Aberrantly increased self-renewal, in combination with the intrinsic growth potential of stem cells, may account for much of what is considered a malignant phenotype.

Thus we could ask; do all organs have stem cells which are organ specific? We know the skin continually reproduces cells, specifically keratinocytes. Colon cancer has a stem cell element[8].

In the paper by Navin and Hicks they present a taxonomy of possible cancer cell propagation. It is worth examining this before driving towards a de4finition. The facts will ultimately determine the definition. They present the following five categories:

1. Clonal: This case is a single cell gets a malignant character and then proceeds with uncontrolled growth. Thus one would expect that each cell in the tumor would reflect this by having genetic homogeneity across any tumor sample. We know that this is clearly not the case.

2. Polyclonal: This is the first case but with a twist. Namely there are several clones clustering together. Thus across any section of the tumor there would be different clones but the clones would be locally consistent. Again this is not the case.

3. Polyclonal via Self Seeding: This is polyclonal but now the different clones appear across the blood stream as separate but homogeneous entities. Again we know that even as hematopoietic spread occurs there is non uniform genetic types.

4. Multiple Mutator: This type is total genetic diversity. Here we go from one genotype to another across almost every cell. Thus the genetic diversity is a maximum complexity. Again we know that this is not the case.

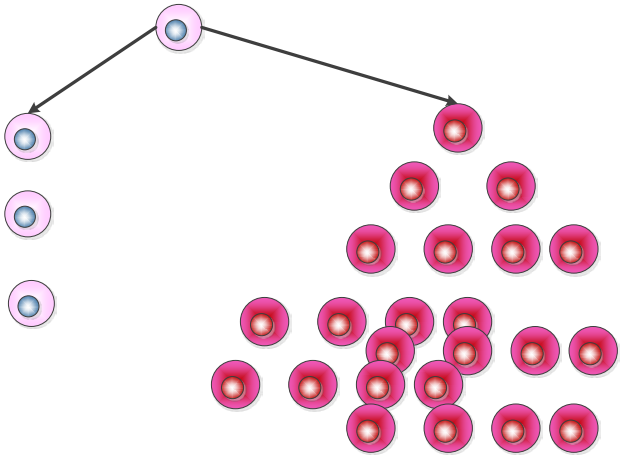
[8] See Rajasekhar p 274.

5. Cancer Stem Cell: This is the hypothesis of a cancer stem cell. Namely a single genotype both self-replicates and also generates a malignant progeny. The progeny cells are basically the same and they can replicate but do not drive the malignancy. Somehow if one were to remove the CSC the other cells would stop. This may be what happens in certain types of HGPIN as we have speculated based upon HGPIN saturation biopsies resulting in return to full benign state.

Now there is a sixth case not discussed by Navin and Hicks which we have proposed and analyzed using limited data. This is the Markov chain model wherein cells have a genotype but the genotype is subject to random changes at random instants, albeit changes driven by location and complexity of the tumor state. We refer the reader to the results elsewhere.

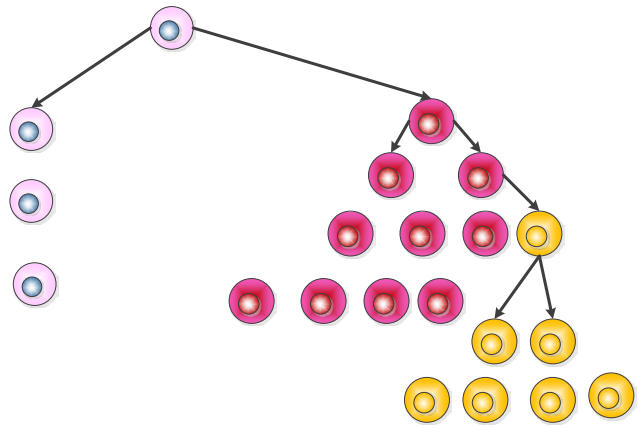
We now demonstrate graphically each of the five cases below:

Case 1: The Clonal Model shows a single mutation and then all subsequent cells are just clones. We have examined this in the case of MDS, Myelodysplastic Syndrome. We know that the malignancy most related to this is AML. We also know that AML appears clonal like but MDS is not the same in all cases. Some have proliferation in red cells while others in platelets. Others may be neutrophil driven. Thus the CSC if there is one is not at the cell base of a core stem cell but at a level above that. Then when AML occurs one may see the CSC moving downward.



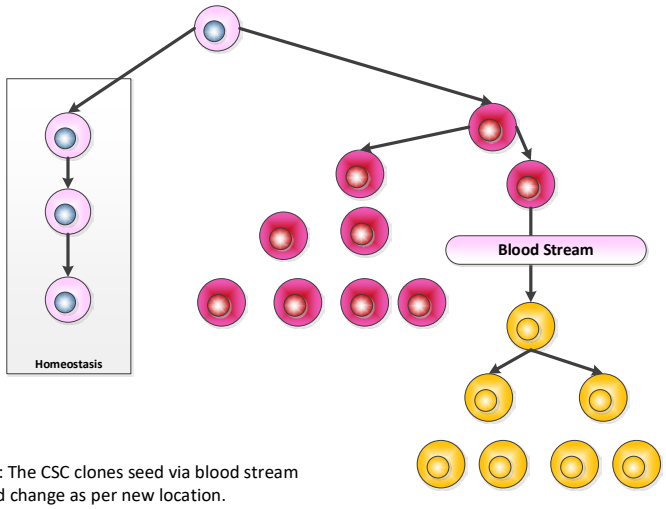
Monoclonal Evolution: Assumes a single malignant clone which continues to replicate

Case 2: Polyclonal. The second model below shows a multiple set of clones. The assumption is that cells continue to mutate but that one an active mutation occurs then the now clone takes off. One would expect to see clusters of common clones.



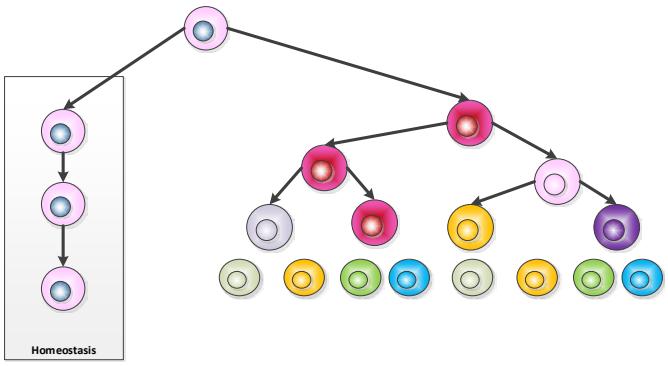
Polyclonal Evolution: Assumes a multiple malignant clones which continues to replicate

Case 3: Self Seeding. The model below is what they call the self-seeding. This is a polyclonal variant where the clone can change as it moves throughout the body.



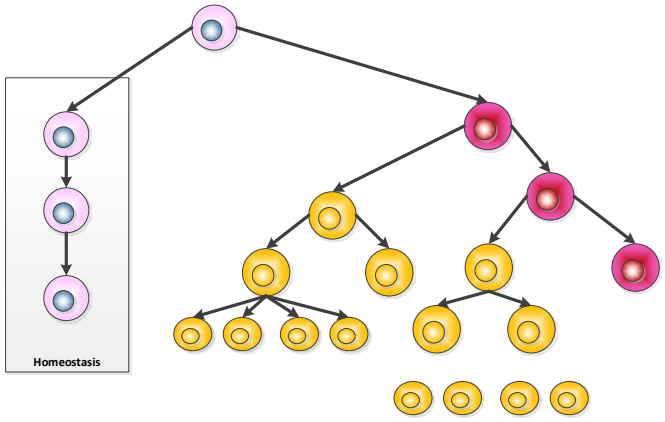
Self Seeding: The CSC clones seed via blood stream and change as per new location.

Case 4: This is the Mutator model wherein cells keep changing is shown below. The end result is a tumor with almost no genomic consistency.



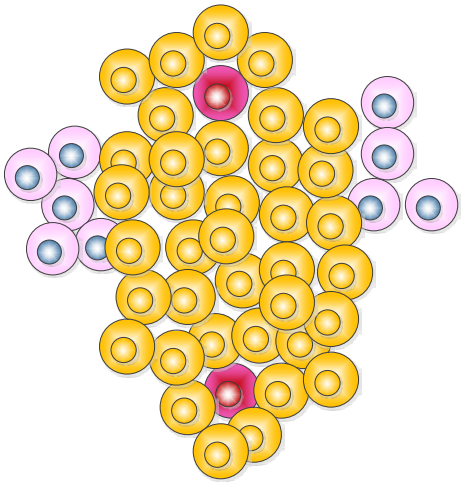
Mutator Phenotype: Generates many variants.

Case 5: This is the case of the CSC. Namely the one cell that starts everything off and keeps it going. We show that below.



Cancer Stem Cell: A single cell self replicates and also produces malignant but non-driving cells.

Thus if we accept the Navin and Hicks description of the CSC we would expect to see a tumor as shown below. Normal cells at a periphery in a normal homeostatic state, then a large collection of non-stem malignant cells (namely cells which can and do proliferate), and a few CSC which somehow drive the process. One could assume the CSCs drive the proliferation of the non-CSC malignant cells. However, that is open for debate. Furthermore, however, if we were to take the CSC away then in most CSC theories the other malignant cells would undergo apoptosis or some form of cell death.



The previous section used a purely logical descriptive approach for CSC classification. As we noted it did have deficiencies that we have explored elsewhere. However, it does not tell us what a CSC is. There are phenomenological

As it is not experimentally feasible to investigate the potential existence of CSCs in human tumors solely on the basis of these theoretical definitions, CSCs are instead defined in practical terms through the use of several functional assays. The most frequently used methodology involves xenotransplantation of flowsorted populations of primary cancer cells into immunodeficient mice.

In this assay, CSCs are defined as a subpopulation of cells within a primary tumor that can initiate tumor formation in mice following transplantation, unlike the remaining tumor cells. Using this assay, early studies identified CSC populations in hematological malignancies, such as the CD34+CD38- population in acute myeloid leukemia.

Similar approaches were subsequently applied to solid tumors, leading to the identification of candidate CSC populations that were prospectively enriched using specific markers in breast (CD44+CD24-Lin-), brain (CD133+) and colon cancers (CD133+). Overall, however, the available evidence supporting the identification of CSCs in solid tumors has been less convincing, at least in part because solid tumor cells exist in a complex microenvironment that is not readily modeled by xenotransplantation.

Wang and Shen then continue to discuss the issue of definitions:

Much of the confusion in the literature arises through inconsistencies in nomenclature within the field. In particular, due to the wide use of xenotransplantation as a functional assay for CSCs, transformed cells that can initiate tumor formation in this assay are often referred to as CSCs in the literature.

However, a ***tumor initiating cell (TIC) represents a different concept from that of a CSC****, as TICs unquestionably exist within tumors and their identification does not by itself imply a*

hierarchical organization of a tumor. Indeed, the majority of cells within a tumor could potentially possess TIC properties and nonetheless follow a clonal evolution model.

Consequently, it is important to distinguish CSCs that have been strictly defined by their position and function within a lineage hierarchy in vivo from CSCs that have been identified as rare TICs in transplantation studies.

A similar confusion arises with respect to the cell of origin for cancer, which corresponds to a normal tissue cell that is the target for the initiating events of tumorigenesis. In principle, a normal adult stem cell could be a logical cell of origin for cancer, as it would retain the ability to self-renew and generate a hierarchy of differentiated lineages within a tumor.

However, it is also possible that a cell of origin could correspond to a downstream progenitor cell or conceivably even a terminally differentiated cell that acquires stem cell properties during oncogenic transformation. For example, both hematopoietic stem cells as well as committed progenitor cells can initiate leukemia after transformation. More recently, activation of canonical Wnt signaling has been shown to transform mouse intestinal stem cells to give rise to adenocarcinomas.

Thus we have as a start three concepts:

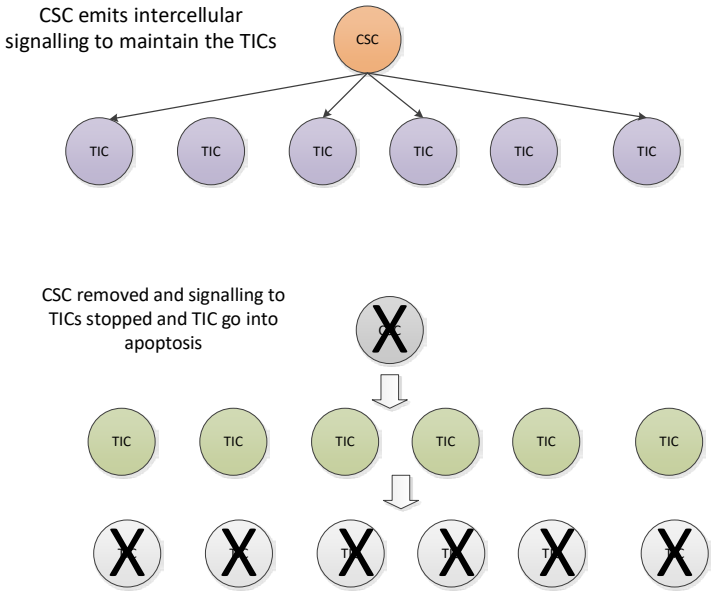
1. Cancer Stem Cell: Also the CSC. This is the self-renewing cell from which the TIC cells arise and which provides the necessary signaling to the TICs to continue their proliferation. Transplanting a CSC will cause a tumor to grow.

2. Tumor Initiating Cell (TIC)[9]: These are cells which are the proliferative cells. They are not the CSC. Transplanting a TIC will result in no growth of a tumor unless accompanied by a CSC.

3. Cell of Origin: Also the Cancer Cell of Origin, CCO. This is the cell from which the CSC was derived. Thus the debate in PCa is often the question; basal or luminal?

This collection leads to a model as shown below:

[9] Weinberg pp 460-463 discusses the CSC and what he terms the Transit Amplifying/Progenitor Cells.



There is a debate about the existence of CSCs for all cancers as well as how one identifies the CSC if indeed it exists for cancers of specific type. We examine this issue again since in much of the literature there are a multiplicity of definitions.

From Nature we have the following definition[10]:

Cancer stem cells are defined as those cells within a tumour that can self-renew and drive tumorigenesis. Rare cancer stem cells have been isolated from a number of human tumours, including hematopoietic, brain, colon and breast cancers. The cancer stem-cell concept has important implications for cancer therapy. However, the generality of the cancer stem-cell hypothesis has also been challenged…

In a similar manner we have the Tumor initiating cell and its relationship to the CSC and the CCO.

From Agarwal et al we have for TIC:

Tumor-initiating cells (TICs), defined by clonal tumor initiation from transplanted cells, have not been analyzed in primary prostate cancers, partly due to the poor transplantation ability of single- cell suspensions of human prostate cancers and low-grade mouse tumors. This may be due to the fragility of fractionated prostate tumor cells, to a high percentage of indolent cells in primary tumors, to a strict requirement for the proper microenvironment, or to other unknown reasons.

Definitions are important. In mathematics and law, the definition will determine the outcome. In engineering we define certain parameters and we design accordingly. If there is a concern that

[10] See: http://www.nature.com/nature/focus/cancerstemcells/

we spend a great deal of time on the definition, that concern should realize that defining something so that it is replicable is a key to scientific study. In cancer studies the term "cancer stem cell" has been introduced but it seems to have been used somewhat loosely.

Definitions should be clear and they should be actionable. Namely the definition should present a way to ascertain through objective measures readily understood by someone trained in the science or art to determine if what is presented satisfies the definition. Namely we should with a good definition know if what we have is a cancer stem cell.

The results below are a sample of what seems to be definitions from the literature. Reading these one can readily see what the complexity is in understanding this topic.

Author	***Definition***
Ailles, and Weissman	*Cancer stem cells (CSCs) are cells that drive tumorigenesis, as well as giving rise to a large population of differentiated progeny that make up the bulk of the tumor, but that lack tumorigenic potential. CSCs have been identified in a variety of human tumors, as assayed by their ability to initiate tumor growth in immunocompromised mice… In addition, specific signaling pathways play a functional role in CSC self-renewal and/or differentiation, and early studies indicate that CSCs are associated with a microenvironmental niche… several important biological properties of CSCs: first, what is the cell of origin for a given tumor? Second, what are the signaling pathways that drive self-renewal and/or differentiation of CSCs? Third, are there molecules uniquely expressed on CSCs, regardless of whether they are functional, that will allow targeted therapies to be developed? Fourth, what are the mechanisms by which CSCs escape conventional therapies and can we defeat these mechanisms?*
Badeux and Tang (in Rajasekhar)	*To fulfill the obligate criteria of a cancer, stem cell (CSC) a cell must be capable of both self-renewal and differentiation, of regenerating and of generating anew…The term cancer stem cell is often replaced by or used synonymously with the phrase tumor initiating cell (TIC).*
Burgess	*Should stem mitotic activity become unregulated or uncontrolled, a tumorigenic and perhaps malignant phenotype may result hence the term cancer stem cell…tumor initiating sells that have malignant properties have been referred to as CSCs…*

Author	*Definition*
Dalerba et al	*Stem cells are defined by three main properties:* *1. differentiation—the ability to give rise to a heterogeneous progeny of cells, which progressively diversify and specialize according to a hierarchical process, constantly replenishing the tissue of short-lived, mature elements;* *2. self-renewal—the ability to form new stem cells with identical, intact potential for proliferation, expansion, and differentiation, thus maintaining the stem cell pool;* *3. homeostatic control—the ability to modulate and balance differentiation and self-renewal according to environmental stimuli and genetic constraints* *Like their normal tissue counterparts, tumors are composed of heterogeneous populations of cells that differ in their apparent state of differentiation. Indeed, the differentiation features of a tumor, morphological and architectural, are the key parameter used in routine clinical practice by surgical pathologists to define a tumor's primary anatomical origin.* *This simple observation suggests that tumors are not mere monoclonal expansions of cells but might actually be akin to "abnormal organs," sustained by a diseased "cancer stem cell" (CSC) population, which is endowed with the ability to self-renew and undergo aberrant differentiation (1, 2). This hypothesis is further reinforced by the fact that cancer is known to result from the accumulation of multiple genetic mutations in a single target cell, sometimes over a period of many years (3). Because stem cells are the only long-lived cells in many tissues, they are the natural candidates in which early transforming mutations may accumulate.*
Dubrovska, A., et al	*One possible explanation for the initial positive response to therapy followed by androgen-refractory disease is that although current therapies eliminate the bulk of the tumor, they fail to eliminate cancer stem cells (CSCs) or tumor-initiating cells (TICs). In fact, it has been argued that many cancers are maintained in a hierarchical organization of rare CSCs, rapidly dividing cells, and differentiated tumor cells; the CSCs are not only a renewable source of tumor cells but are also a source of tumor resistance leading to tumor recurrence, metastasis, and tumor progression. Support for this hypothesis came with the identification of TICs in leukemia in 1994 and, subsequently, in a variety of cancers, including solid tumors. In addition, cancer cell lines have been shown to harbor cancer stem-like cells and are a promising model for CSC research because these progenitors can be readily expanded under anchorage independent (sphere formation) serum-free conditions*

Author	***Definition***
Fang et al,	*Recent studies suggest that cancer can arise from a cancer stem cell (CSC), a tumor-initiating cell that has properties similar to those of stem cells. CSCs have been identified in several malignancies, including those of blood, brain, and breast.*
Hurt et al	*The cancer stem cell hypothesis suggests the existence of a small subpopulation of cells within the tumour that give rise to differentiated tumour cells. It is thought that the cancer stem cells survive conventional treatment to later re-emerge more resistant to therapy. To date, putative cancer stem cells have been identified in blood, brain, breast, lung, skin, pancreas, colon, and prostate*
Jordan et al	*Stem cells have three distinctive properties: self-renewal (i.e., at cell division, one or both daughter cells retain the same biologic properties as the parent cell), the capability to develop into multiple lineages, and the potential to proliferate extensively. The combination of these three properties makes stem cells unique. The attribute of self-renewal is especially notable, because its subversion is highly relevant to oncogenesis and malignancy. Aberrantly increased self-renewal, in combination with the intrinsic growth potential of stem cells, may account for much of what is considered a malignant phenotype. Biologically distinct and relatively rare populations of "tumor-initiating" cells have been identified in cancers of the hematopoietic system, brain, and breast. Cells of this type have the capacity for self-renewal, the potential to develop into any cell in the overall tumor population, and the proliferative ability to drive continued expansion of the population of malignant cells. Accordingly, the properties of tumor-initiating cells closely parallel the three features that define normal stem cells. Malignant cells with these functional properties have been termed "cancer stem cells"*
Lawson and Witte	*Two theories were proposed to explain this paradox. The stochastic theory suggested that all cancer cells are equally malignant but only clones that randomly possess favorable biological properties will grow upon transplantation. An alternative theory predicted that tumors are hierarchical like normal tissues and only the rare subpopulation of cells at the pinnacle of that hierarchy have the unique biological properties necessary for tumor initiation (8, 9). Studies by John Dick and colleagues provided evidence for the hierarchy model. This group demonstrated that only the small subpopulation (0.1%–1.0%) of Lin–CD34+CD38– cells within human acute myelogenous leukemia samples were capable of initiating disease when transplanted into immune-deficient mice (10). These cells possessed the same antigenic profile as normal human HSCs, which are at the pinnacle of the normal hematopoietic hierarchy. This population also had the unique capacity to selfrenew to propagate the disease as well as differentiate to produce the many leukemic cell types represented in the original leukemia. Since these cancer cells possess properties unique to normal tissue stem cells, they have been termed "cancer stem cells" (CSCs).*

Author	***Definition***
Lobo et al	*Stem cell: a primitive cell defined by its capacity to self-renew and differentiate into at least one mature cell type* *Cancer stem cell: a self-renewing cell within a tumor that has the capacity to regenerate the phenotypic diversity of the original tumor*
NCI	*The theory of the cancer stem cell (CSC) has generated as much excitement and optimism as perhaps any area of cancer research over the last decade. Biologically, the theory goes, these cells are distinct from the other cells that form the bulk of a tumor in that they can self-perpetuate and produce progenitor cells, the way that traditional stem cells do. The progenitors' job is then to repopulate tumor cells eradicated by treatments such as chemotherapy or radiation. But for all the attention and fanfare CSC research has received, the findings reported to date are far from clear-cut, investigators acknowledge. For example, most of the studies that have identified human CSCs have used mouse xenograft assays and cells from only a small number of human tumor samples, making it difficult to draw firm conclusions. In addition, other researchers haven't always been able to replicate initially reported findings. And while these tumor-initiating cells, as they are also called, have been described as being a rare class, several studies have found that the number of cells that can form tumors in these mouse experiments is actually quite large, suggesting that perhaps CSCs aren't such a privileged breed.*
Pavlovic and Balint	*As the stem cells that created the tumor to begin with are so few in number, scans following treatment usually fail to identify populations of CSCs in this limited population….*[11]
Perego et al	*Although there is no definitive consensus on the phenotype and frequency of CSCs in the majority of human tumors, much experimental evidence supports the contentions that many tumors of both epithelial and nonepithelial origin have operationally defined CSCs (cells able to propagate tumors in immunodeficient mice) and that the presence of these CSCs affects tumor biology.*
Rajasekhar	*The "cancer stem cell model" CSC …envisions tumors as "pathological organs" sustained in their aberrant growth by a mutated population of stem cells, in which normal homeostatic controls on tissue expansion have been lost.*
Roesch et al	*The CSC concept postulates a unidirectional hierarchy of tumor cells…According to the traditional CSC concept, tumor initiation is regarded as an exclusive characteristic of CSCs*

[11] This book is near incomprehensible. It is impossible to find a definition, only secondary referral characteristics at best!

Author	*Definition*
Rosen and Jordan	*Thus, the CSC paradigm refers to the ability of a subpopulation of cancer cells to initiate tumorigenesis by undergoing self-renewal and -differentiation, like normal stem cells, whereas the remaining majority of the cells are more "differentiated" and lack these properties.*
Soltysova, et al	*Normal stem cells in the adult organism are responsible for tissue renewal and repair of aged or damaged tissue. A substantial characteristic of stem cells is their ability for self-renewal without loss of proliferation capacity with each cell division. The stem cells are immortal, and rather resistant to action of drugs. They are able to differentiate and form specific types of tissue due to the influence of microenvironmental and some other factors. Stem cells divide asymmetrically producing two daughter cells – one is a new stem cell and the second is progenitor cell, which has the ability for differentiation and proliferation, but not the capability for self-renewal.* *Cancer stem cells are in many aspects similar to the stem cells. It has been proven that tumor cells are heterogeneous comprising rare tumor initiating cells and abundant non-tumor initiating cells. Tumor initiating cells – cancer stem cells have the ability of self-renewal and proliferation, are resistant to drugs, and express typical markers of stem cells. It is not clear whether cancer stem cells originate from normal stem cells in consequence of genetic and epigenetic changes and/or by redifferentiation from somatic tumor cells to the stem-like cells. Probably both mechanisms are involved in the origin of cancer stem cells. Dysregulation of stem cell self-renewal is a likely requirement for the development of cancer. Isolation and identification of cancer stem cells in human tumors and in tumor cell lines has been successful.*

Author	***Definition***
Visvader	*It is important to note that the cell of origin, the normal cell that acquires the first cancer-promoting mutation(s), is not necessarily related to the cancer stem cell (CSC), the cellular subset within the tumour that uniquely sustains malignant growth. That is, the cell-of-origin and CSC concepts refer to cancer-initiating cells and cancer-propagating cells, respectively (Fig. 1). Although the tumourinitiating cell and the CSC have been used interchangeably, the tumour-initiating cell more aptly denotes the cell of origin. There is considerable evidence that several diverse cancers, both leukaemias and solid tumours, are hierarchically organized and sustained by a subpopulation of self-renewing cells that can generate the full repertoire of tumour cells (both tumorigenic and non-tumorigenic cells)1. The cell of origin, the nature of the mutations acquired, and/ or the differentiation potential of the cancer cells are likely to determine whether a cancer follows a CSC model. In most instances, the phenotype of the cell of origin may differ substantially from that of the CSC.* *Normal cellular hierarchy comprising stem cells that progressively generate common and more restricted progenitor cells, yielding all the mature cell types that constitute a particular tissue. Although the cell of origin for a particular tumour could be an early precursor cell such as a common progenitor, the accumulation of further epigenetic mutations by a cell within the aberrant population (in this case expanded) during neoplastic progression may result in the emergence of a CSC. In this model, only the CSCs (and not other tumour cells) are capable of sustaining tumorigenesis. Thus, the cell of origin, in which tumorigenesis is initiated, may be distinct from the CSC, which propagates the tumour.*
Wang and Shen	*In its strictest form, the CSC model posits a hierarchical organization of tumors, with cancer stem cells at the top of the lineage hierarchy being capable of indefinite self-renewal, unlike their progeny, which undergoes an epigenetic program of differentiation and loss of tumorigenicity In this view, rare CSCs may represent the driving force of tumor malignancy, and therefore effective treatment could be achieved by specific targeting of the CSC population. In contrast, the stochastic (clonal) evolution model proposes that most of the cancer cells within a tumor are highly tumorigenic and possess different genetic or epigenetic properties Consequently, it is important to distinguish CSCs that have been strictly defined by their position and function within a lineage hierarchy in vivo from CSCs that have been identified as rare TICs in transplantation studies.*
Weinberg p 462	*…the tumor initiating cell, often termed a cancer stem cell (CSC), is self-renewing and has the ability to generate the countless neoplastic progeny that constitute a tumor. While the CSC and its progeny are genetically identical, the progeny, because they have lost self-renewing ability, have also lost tumor initiating ability.*

It does not take an extensive reading to see the overlap of ideas. Ideas of function and action.

There is often a set of confusion regarding which cell does what. As we have discussed above the CSC is the driving cell for malignant growth. In contrast the CCO is the cell that originally underwent transformation. Is there a connection between them? Clearly the CSC must be some derivative of the CCO. But the CCO is reflective of where the initial genetic alteration occurred. As Tang et al state regarding CSC and CCO we have:

A tumor originates from a normal cell that has undergone tumorigenic transformation as a result of genetic mutations.

This transformed cell is the cell-of-origin for the tumor.

In contrast, an established clinical tumor is sustained by subpopulations of self-renewing cancer cells operationally called cancer stem cells (CSC) that can generate, intraclonally, both tumorigenic and nontumorigenic cells.

Identifying and characterizing tumor cell-of-origin and CSCs should help elucidate tumor cell heterogeneity, which, in turn, should help understand tumor cell responses to clinical treatments, drug resistance, tumor relapse, and metastatic spread. Both tumor transplantation and lineage-tracing assays have been helpful in characterizing these cancer cell populations, although each system has its strengths and caveats.

In this article, we briefly review and summarize advantages and limitations of both assays in support of a combinatorial approach to accurately define the roles of both cancer-initiating and cancer-propagating cells. As an aside, we also wish to clarify the definitions of cancer cell-of-origin and CSCs, which are often interchangeably used by mistake.

The CCO, cancer cell of origin, is distinct from the CSC. Below we depict a typical test. We select a set of tumor cells. We then mark them with some appropriate marker so that we can separate CSC and TIC cells as well as whatever else is in the mix. The markers are often based on what proteins each cell expresses. The we transplant them to a mouse and examine the result. If we have a CSC, then the tumor regrows. If TIC or benign cells, then no growth.

The above graphic is the approach often used. Namely take a cell which may be expressing a specific surface marker and then implant it in a mouse and observe the result. If the cell replicates the human tumor, then we have "found" the CSC. It is not clear that mice may not be primed for this. It is not clear how coincidental this may be. There should be a body of justification which is much more extensive.

4.2 A THOUGHT EXPERIMENT

The cancer stem cell concept is somewhat akin to the overall stem cell. Simply, a Cancer Stem Cell appears to be as a concept a single stem cell with some well-defined DNA structure which becomes capable during mitosis of;

(i) regenerating itself consistently in some near immortal manner,

(ii) while simultaneously generating another cell which is different from itself and which itself may duplicate itself exactly, subject to random genetic changes, and

(iii) that such CSC if transplanted alone to some unaffected carrier will regenerate the tumor from which it was extracted.

This definition is an amalgam of the many attempts to define such a cell.

We know that such a process as the stem cell, albeit benign, appears to exist in hematopoiesis. Also it has been argued that such a cell is the basis for a variety of hematopoietic malignancies, such as MDS. MDS is especially interesting since it occurs not with the hematopoietic stem cell but somewhere along the line such a myelo or lympho line and that it involves methylation yet there is a CSC like behavior.

Let us begin with some facts:

1. All somatic cells have the same DNA. This is almost true. There are exceptions as follows:

a. There may have been some somatic mutation or translocation.

b. There may be some epigenetic changes due to methylation or miRNAs for example.

2. Mitosis of a single cell produces two identical offspring. There are some differences however:

a. First what do we mean by identical? They clearly have the same DNA but some DNA may be expressed slightly differently. Why is one cell expressing DNA differently than the other? Why is the other cell, if that be the case, working identically as its parent cell? Are the previous statements true?

b. Phenotypically there may be a significant difference in the cells.

3. A stem cell is defined in a certain manner. Essentially it is a self-replicating cell that can give rise to itself by definition and to other cells which may become mature cells in some terminal sense. However:

a. How does one identify a stem cell? Generally, it has been identified as a cell which when transplanted to a genetically primed target, a mouse for example, that it generates and reproduces the initial cancer. Furthermore, if it is silenced or removed the cancer ceases.

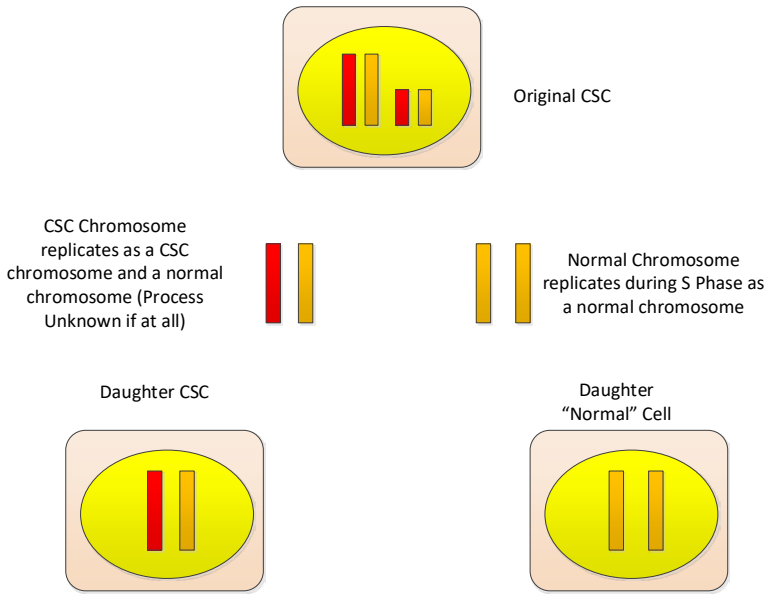
4. A cell of origin is a cell from which the original cancer arises. Yet:

a. What do we mean by the original cancer?

b. What is the relationship to the CSC?

5. A cancer stem cell, CSC, is a cell which can be defined as a self-replicating cell which also produces a second type of cell which is less self-replicating but which becomes the body of a tumor. The CSC somehow using the same DNA manages to go through the cell cycle yet produces two phenotypical cells which are also genotypically different in their expression albeit genotypically the same in toto.

We try to demonstrate this artifact below. One must note that there is as of yet no physical basis for this claim. It is merely a thought experiment.



What do we have above? We have the following:

1. A cell with DNA that has somehow had some malignant alteration in two of the chromosomes. We have one chromosome marked as red which renders some semblance of immortality and a second chromosome which is marked orange which renders excess growth, albeit with limited mortality. Homologous orange chromosome cells are aggressive growers but can die off.

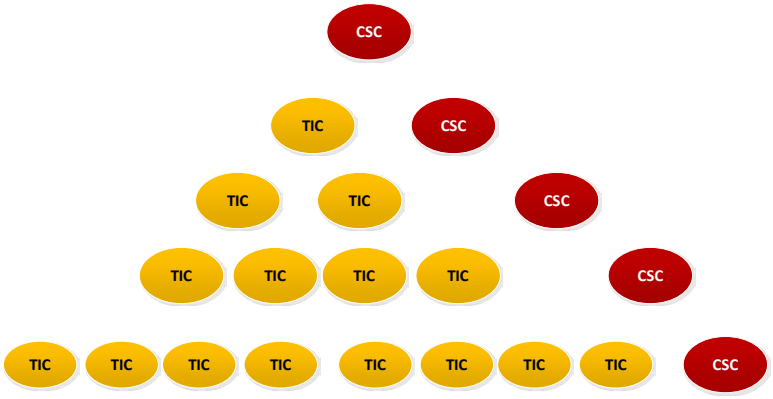
2. Now somehow, we really do not know but just posit a result, which makes this a thought experiment, the CSC goes through mitosis and produces two cells; a duplicate of itself and a daughter cell with homologous orange chromosomes.

3. The homologous cell goes on to replicate and then can go into apoptosis and die off.

4. The CSC can replicate again.

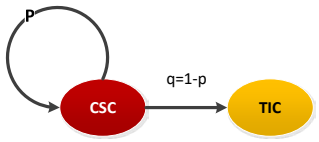
5. The CSC can replicate in one of two ways. First it can be deterministic, namely one CSC yields one CSC and a homologous cell. Second one CSC can regenerate itself with some probability and produce a homologous cell with another probability. The latter is the stochastic case.

We demonstrate the deterministic below:



Deterministic: The CSC replicates itself each time and the TIC also replicates but it doubles. Thus we see a single CSC while the TICs double.

We demonstrate the stochastic as follows:



Stochastic: The CSC can split into another CSC with a probability p or a TIC with probability q. If, for example, it splits into 2 TIC then the CSC could die off. It also could split into two CSC which would then cause added growth.

With the above model one can determine the distribution of cells as a function of time. For a linear progression the split is always 50:50 and otherwise we would have a probability that the CSC itself could extinguish. Even if p approaches 1.0. and never really reaches it then there is a minute but possible extinction. We do have examples of tumor regression. The classic case is in melanoma and in Rosenberg's early observations. We also know that in the case of HGPIN, that most likely we have some form of stem cell and that HGPIN also regresses in a finite number of cases[12].

A great deal of work has been done examining the dynamics of CSCs. Part of that efforts pertains to establishing some means to identify them. We examine a small subset of the models here but there are many studies worth examining. Fundamentally the studies all seem to reflect the approach which starts with a stem cell and examines the products resulting therefrom. We

[12] It is worth reading some of the cases we have discussed in the White Papers. There is a recent case where we saw total extinction.

have argued elsewhere and summarize latter herein that there is an alternative approach which frankly eschews the CSC and examines a collection of cells each sub-collection having a specific genetic expression state. That approach only looks at the cancer as a separate organism from the host and tries to understand it holistically as a spatio-temporal collection of interdependent genetic expression states evolving over time.

Many authors have examined the mathematical dynamics of the stem cell and the CSC. Stukalin et al have developed models for the fluctuations in cell populations. In a sense this is always a significant issue since the CSC growth is complex and does not reflect a simple deterministic model. Dhawan et al have examined the tumor control mechanisms in dynamic CSC environments. This is one of many ways that the CSM paradigm could be used in the control of cancers. Shahriyari et al have examined mathematical models for the stochastic dynamics of the CSC environment. They look at multiple mutations and effects on non-symmetric changes. Zhang and Wolynes use the many-body paradigm to explore stability points in complex CSC models. These are but a few of the approaches taken in modelling the CSC environment.

4.3 SOME BASICS

Let us begin with some simple fundamentals. As Dingli and Pacheco note:

Tissues have evolved an architecture where most cells have a relatively short lifetime and undergo continuous turnover, and this mitigates the accumulation and retention of mutant cells.

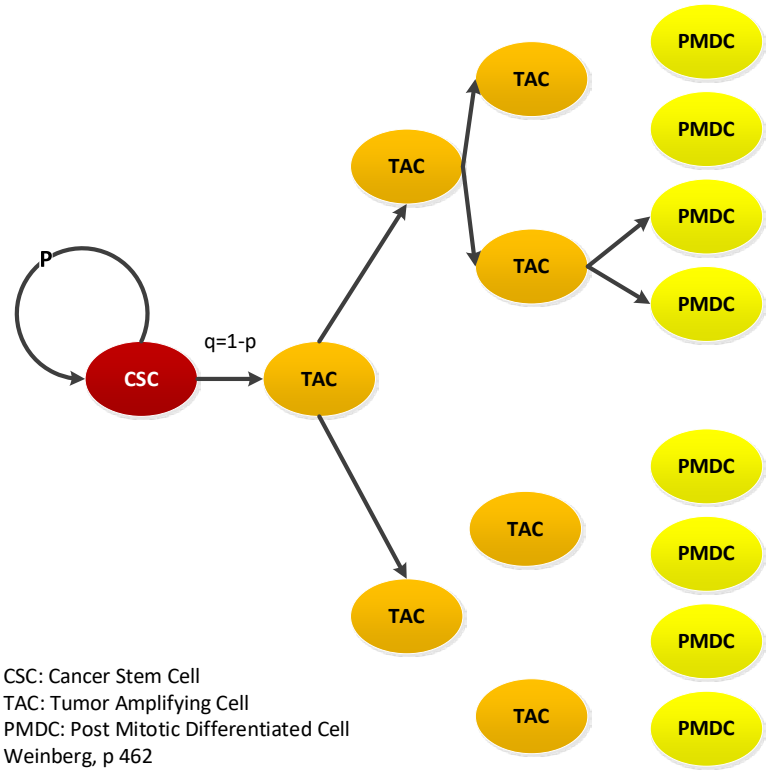
At the root of this process are the stem cells that are able to maintain tissue integrity because of a dual phenotypic characteristic: self-renewal and production of progeny that can differentiate into various cell lineages that together constitute tissues and organs.

One can visualize tissues as having a tree-like organization of cells with stem cells at one extreme and mature, non-dividing cells at the other extreme.

Intermediate cells divide, often at relatively high rates, but live for relatively short periods of time. Although mutations can occur at every level of this cell hierarchy, the relatively short lifetime of more mature cell stages means that, in effect, the real risk of long-lasting oncogenic mutations is restricted to the small population of stem cells and early progenitor cells that maintain a given tissue.

This, in turn, effectively reduces the probability of the occurrence of mutations, given the small population of cells at risk, despite the fact that a mutation arising in a stem cell can persist for a long time. It is important to point out that the relevance of a mutation is cell context-dependent – a mutation in a gene that is not expressed in a cell is of no consequence to that cell but expression of the gene in more committed cells, downstream of the cell that is the source of the mutation, may lead to a phenotype associated with disease.

From Weinberg we have the following model which reflects the above:



Note that in the above model we have the long lasting CSC and then we have proliferating intermediaries and ultimately the non-proliferating end stage cells. As the above authors note that since this is stochastic then there is a multiplicity of end states. At one extreme the CSC may actually die off, it may not reproduce and thus the cancer may just regress. We have argued that in certain cases of HGPIN followed by high saturation prostate biopsy that one may actually capture the CSC in a single core and thus deprive the nascent malignancy of its growth potential. Also one could imagine the immune system performing a similar function.

Bogdan et al report:

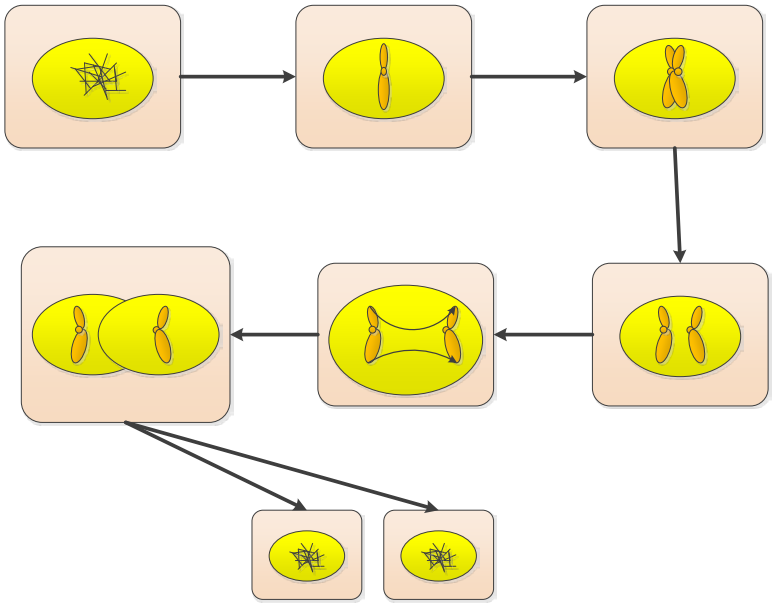
Stem cell division times exhibit non-stationary behavior. Besides the heterogeneous structure of stem cells population, we also observe that the empirical PDF estimated from stem cell DTs exhibits a pronounced time dependent behavior…

Stem cell growth rates possesses multi-fractal characteristics. For a comprehensive investigation of the heteroscedastic dynamics of stem cell growth, we investigate the relationship between the higher order moments of stem cells dynamics and their order; we also estimate both the multi-fractal spectrum and generalized Hurst exponent function

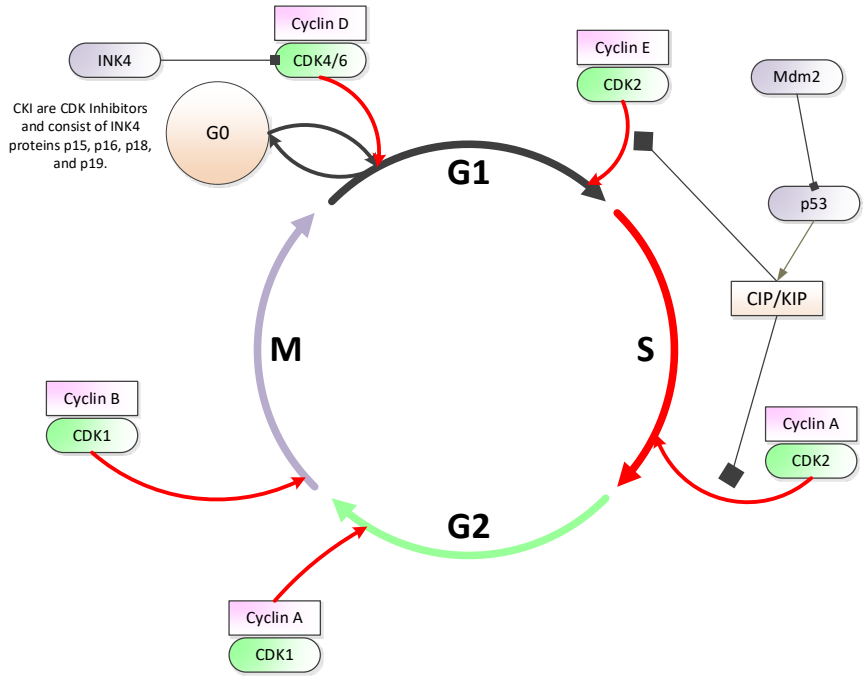
4.4 The Cell Cycle

A fundamental element of the understanding of cancer dynamics and the issues related to CSCs is the cell cycle itself. We start with a simplified description of mitosis. The intent here is not to

present mitosis which is well documented in a multiplicity of places but to place a focus on some of the issues of the CSC. The simplified cycle is below:



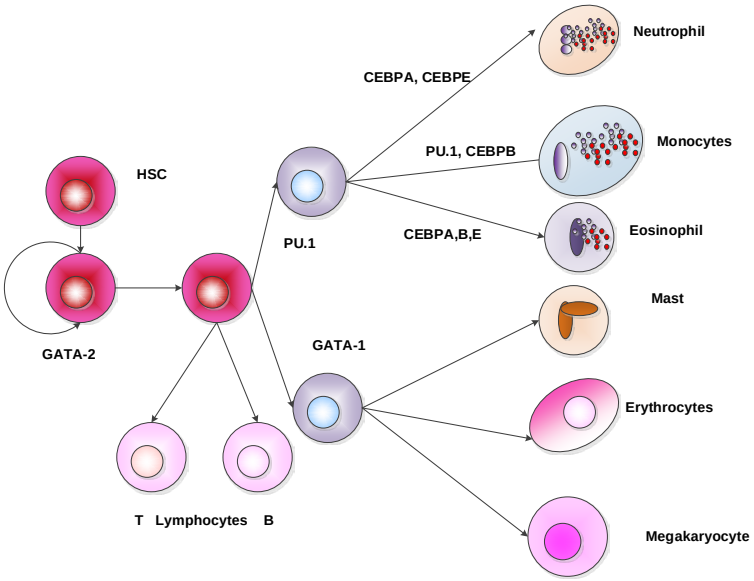
Now note the key step is in the reproduction or duplication of the DNA in the S phase. That is the last step on the top, we see a doubling of the chromosome. We detail the cycle below for reference.



Now let us consider two processes in which this occurs:

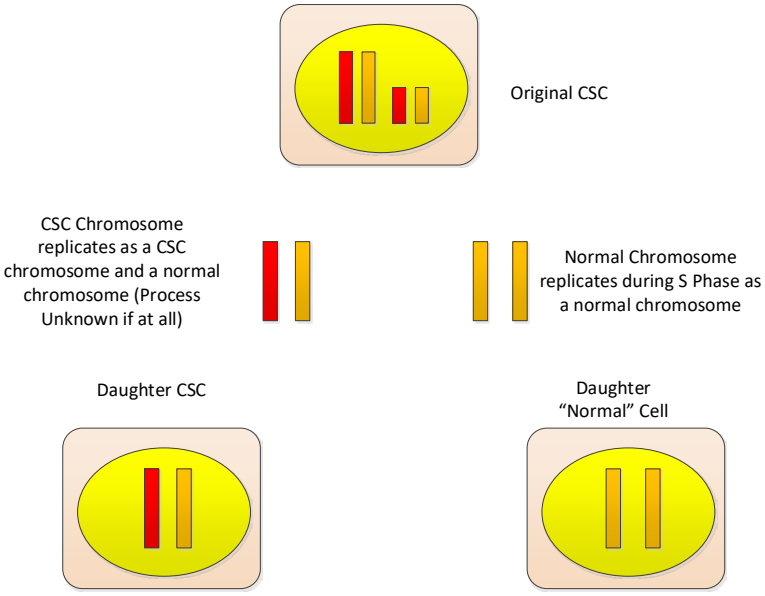
1. Hematopoiesis: As the stem cell for the various blood lines evolve as shown below we have a cell move along but it changes based on what its local environment presents. The stem cell in the

bone produces two stem cells, one which stays put, I am assuming a deterministic model, and another moves, and as it moves it encounters ligands that attach to receptors and the cell begins to change. As it changes it goes through mitosis again perhaps and it again encounters more ligands and changes some more.



This process continues until complete maturity.

2. CSC: This model is problematic Recall the assumption below:



There is the issue of recreating the CSC while also creating a new cell where the S phase appears to have some asymmetry. This is problematic. There is no well-known process whereby this can occur.

4.5 IDENTIFYING CSCS

There are currently several ways to identify CSCs. The primary one is via cell surface markers and in the case of PCa one specific one is CD44. Karsten and Goletz present a recent review of a collection of such markers. As they note:

In recent years' considerable effort has been invested in the detection and characterization of stem cell markers. The result is that there are now an overwhelming and steadily increasing number of such marker molecules. Some markers are indeed more or less specific for different types of stem cells, for example, markers that differentiate embryonic from adult stem cells or pluripotent from progenitor cells. With the exception of pluripotent embryonic stem cells all other stem cells carry, in addition, lineage-specific markers.

Stem cells are also defined by the absence of certain markers. Contemplating these data, several questions arise. First, as already mentioned, almost all markers of normal stem cells are also found on cancer stem cells. This, of course, poses a problem with respect to their potential use as therapeutic targets. Ectopic (non-lineage) expression of stem cell markers on cancer cells does not resolve the therapeutic dilemma. Currently the best option for a therapeutic target would be to rely on onco-fetal stem cell markers which are not expressed on normal adult stem cells. Otherwise there is at present no clear-cut distinction available between normal and cancer stem cell markers. Even at the level of regulatory miRNA clusters, identical patterns were observed

They continue:

These data and other more general considerations led us to propose the following hypothesis.

1. During the process of malignant transformation from a normal stem or progenitor cell to a cancer stem cell, stem cell glycoprotein markers undergo alterations in their glycosylation.

2. As a consequence, cancer stem cells carry cancer specific glycans.

3. This appears to be a selective process. Accordingly, these cancer-specific glycans are CSC makers.

4. Changes in stem cell marker glycosylation contribute to the altered biological behavior of these cells.

In brief, we propose that cancer stem cell markers differ from their normal counterparts by the expression of tumor-specific glycans.

We have seen the glycan presence previously. But the change in glycosylation may be a change in energy utilization which we have also seen in the Warburg process. Thus the glycan markers may logically be targeted as markers. The logic and data in this paper may add more to the understanding of the CSC dynamics.

4.6 PATHWAY ISSUES

We briefly examine some of the key pathways that have been argued as critical in the CSC evolution. Although we present them we however do not attribute anything specific to them herein.

As Zhang et al note:

IPA uncovered important signaling pathways enriched in basal cells including

1. *TGF-b,*
2. *NOTCH,*
3. *WNT/TCF,*
4. *IGF,*
5. *FGF,*
6. *STAT3/ IL6 and others.*

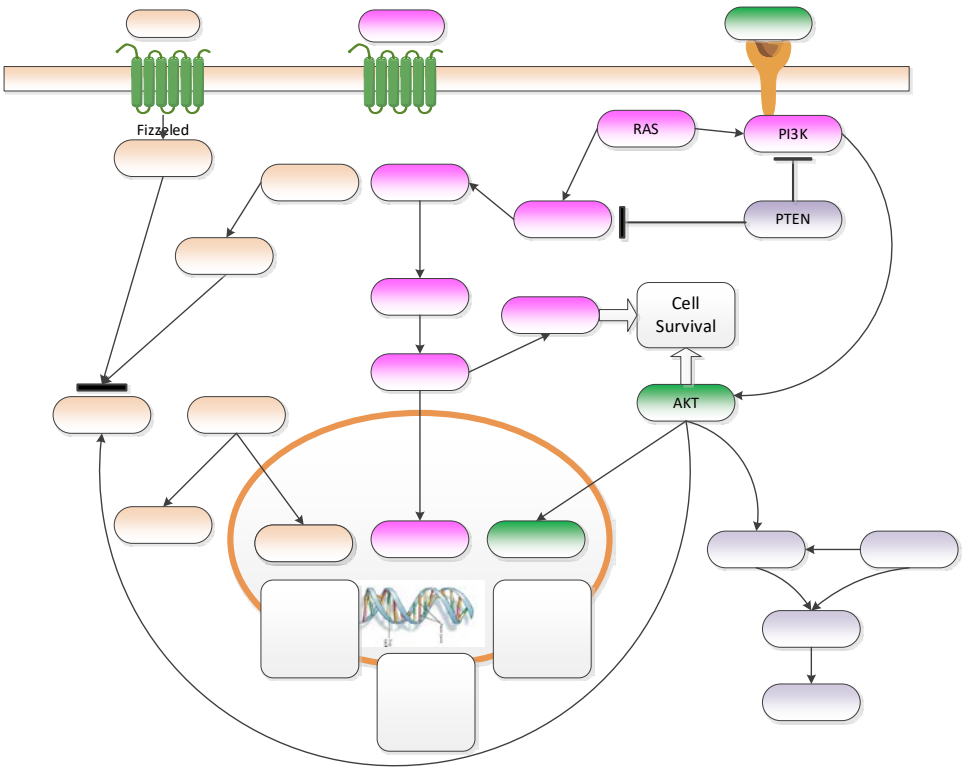
For instance, immunofluorescence of FGFR3 validated our RNA-Seq data and revealed its expression preferentially in the basal layer. We systematically investigated some of these pathways in regulating primary basal stem/progenitor activities.

Given that each pathway has a large number of components, we first used the pathway-specific pharmacological inhibitors to interrogate their roles in regulating basal cell activity. For pathways of particular interest, small interfering RNA (siRNA)-mediated knock-down experiments were performed to validate the inhibitor results.

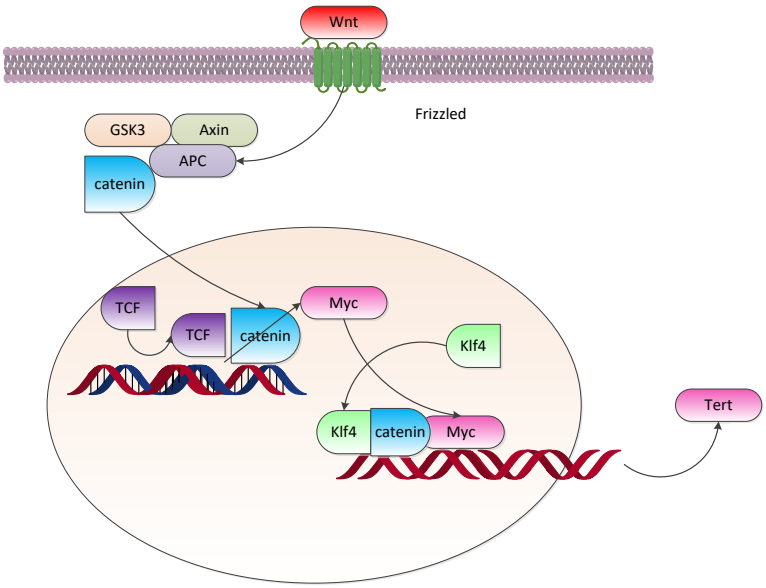
4.6.1 WNT

We briefly re-examine each of these. First we show the WNT pathways below. This is a well know process and we have examined it extensively previously[13].

[13] Specifically see the reference by Goss and Kahn.



Signaling pathways in the cells have been a major focus on study for the past decade or so. The focus generally has been on what protein or gene influences what other protein or gene. A recent article in Science presents some interesting work on Wnt and TERT[14].



[14] http://science.sciencemag.org/content/336/6088/1519

Wnt is an extra cellular signaling protein and it attaches to Frizzled a receptor and sets off a cascade that moves B catenin into the nucleus and generates Myc which is a transcription protein with together with catenin and other transcription proteins generates Tert from TERT.

To quote from NCBI[15]:

Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse transcriptase activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis.

As the Science article states:

Maintaining the length of telomere, the ends of chromosomes, is essential for all cells that divide many times. The enzyme telomerase lengthens these ends, counterbalancing their shortening that occurs each time chromosomes are copied. Telomerase is essential for cell viability, and loss of its function from the loss of only one of two copies of the encoding gene can lead to the failure of stem cell renewal that is seen in premature aging conditions such as dyskeratosis congenita, aplastic anemia, and pulmonary fibrosis. Conversely, telomerase activity is increased in many cancers and may be required for cancer cells to maintain their telomere length...

They continue is a rather interesting wording:

Because of the importance of telomerase expression, the signaling pathways that control TERT transcription have been extensively studied. Remarkably, many different transcription factors, including c-Myc, Sp1, nuclear factor of activated T cells (NFAT), activating protein 2B, nuclear factor κB (NF-κB), Myb, activating transcription factor, nuclear factor 1 (NF1), and the estrogen receptor (ER), bind to the 330–base pair minimal TERT promoter and regulate transcription. In addition, a number of negative regulators bind the TERT promoter, including CTCF, elongation factor 2, p53, Ets, Mad1, Men1, and Wt1. Adding β-catenin and Klf4 to the many regulators that bind the TERT promoter is like adding one more guest to a crowded table at a dinner party.

They conclude:

It is reasonable to propose that Wnt regulates TERT given that Wnt signaling plays an essential role in stem cell self-renewal and that TERT is needed for the long-term growth of stem cells. TERT regulation seems to require not one, but two master transcriptional regulators to assure that there is neither too much, which may allow the growth of cancer cells, nor too little, which might lead to stem cell failure. The finding by Hoffmeyer et al. that both β-catenin and Klf4 are required to activate TERT expression puts the horse (Wnt) before the cart (TERT) and provides a foundation for linking telomerase levels and self-renewal.

[15] http://www.ncbi.nlm.nih.gov/gene/7015

The observation of the inter-cellular signaling with Wnt and its control over TERT and the telomere process is quite interesting. This may be an interesting way to incorporate many of the Turing models we have been discussing as well.

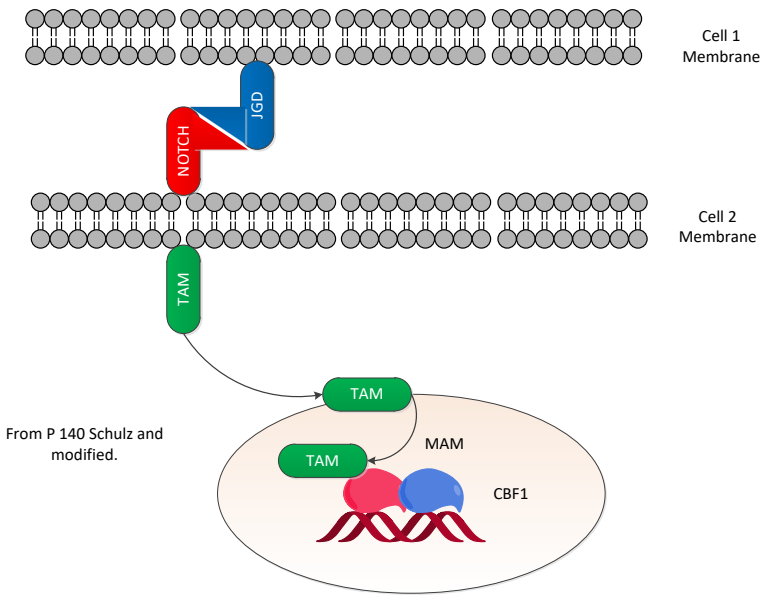
4.6.2 NOTCH

Notched is a bit of an amalgam of the above discussion. The notched pathway is characterized as follows.

The notch protein sits like a trigger spanning the cell membrane, with part of it inside and part outside. Ligand proteins binding to the extracellular domain induce proteolytic cleavage and release of the intracellular domain, which enters the cell nucleus to alter gene expression. The notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life. Notch signaling also has a role in the following processes:

1. neuronal function and development
2. stabilization of arterial endothelial fate and angiogenesis
3. regulation of crucial cell communication events between endocardium and myocardium during both the formation of the valve primordial and ventricular development and differentiation
4. cardiac valve homeostasis, as well as implications in other human disorders involving the cardiovascular system
5. timely cell lineage specification of both endocrine and exocrine pancreas
6. influencing of binary fate decisions of cells that must choose between the secretory and absorptive lineages in the gut
7. expansion of the hematopoietic stem cell compartment during bone development and participation in commitment to the osteoblastic lineage, suggesting a potential therapeutic role for notch in bone regeneration and osteoporosis
8. T cell lineage commitment from common lymphoid precursor
9. regulation of cell-fate decision in mammary glands at several distinct development stages
10. possibly some non-nuclear mechanisms, such as control of the actin cytoskeleton through the tyrosine kinase Ab

We demonstrate Notched and its counterpart Jagged in the following Figure. On the cell surface we have Notched and on the other cell surface we have Jagged. When they bond, in a sense as surface proteins but with a communicating capability, Notched release or activates Tam which is a transcription factor facilitator.



Notch signaling is dysregulated in many cancers.

4.6.3 FGF

FGFR is a Receptor and this gene encodes a member of the fibroblast growth factor receptor (FGFR) family, with its amino acid sequence being highly conserved between members and among divergent species. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein would consist of an extracellular region, composed of three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment and a cytoplasmic tyrosine kinase domain. The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. This particular family member binds acidic and basic fibroblast growth hormone and plays a role in bone development and maintenance.

As we have noted elsewhere FGF is one of many such receptors as shown below:

Models	Genes regulated	Prostate phenotype
Hormone receptors	Androgen receptor	HGPIN
	Retinoic acid receptor α/γ	Squamous metaplasia and pre-neoplastic lesions
	Estrogen receptorα/β	No marked phenotype
Growth factors and receptors	FGF8b	HGPIN
	FGFreceptor1	PIN with reversible hyperplasia
	FGF7	Prostate epithelial dysplasia
	FGFR2iiib	Hyperplasia/dysplasia
	IGF-1	PIN and spontaneous tumor growth
	TGFR-β	PIN and invasive adenocarcinoma
	HER-2/Neu	PIN and invasive carcinoma
Tumor suppressors, cell cycle, and signaling pathways	p53Rb	PIN with reduced apoptotic potential Focal hyperplasia
	Nkx3.1	Hyperplasia followed by PIN
	H-Ras	LGPIN and intestinal metaplasia
	APC	PIN and invasive adenocarcinoma
	Pten	PIN and metastatic adenocarcinoma
	Bcl-2	No overt phenotype
	Akt-1	Focal regions of PIN
	C-MYC	PIN and locally invasive adenocarcinoma
Genomic instability	Eco RI c-fos	HGPIN No significant pathology
Composite transgenic mice	Ink4a/Arf+/−/Pten+/−	Rapid growth of PIN lesion
	Nkx3.1/Pten	PIN and metastatic spread of invasive tumors to lymph nodes
	Pten+/−/Akt1−/−	Akt1−/− repressed prostate tumor growth
	Pten+/−/p27kip1−/−	Rapid progression of invasive carcinoma
	Pten−/−/p53−/−	Early onset of invasive tumors
	PTEN+/−/TRAMP	Increased rate of tumor development
	P53−/−/Rb−/−	Highly metastatic adenocarcinoma
	Pten+/−/FGF8b	Metastatic adenocarcinoma
	Bcl-2/TRAMP	Multi step prostate carcinogenesis

4.6.4 TGF-β/EMT

As we had noted previously[16]:

TGFB1: This gene encodes a member of the transforming growth factor beta (TGFB) family of cytokines, which are multifunctional peptides that regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types. Many cells have TGFB receptors, and the protein positively and negatively regulates many other growth factors. The secreted protein is cleaved into a latency associated peptide (LAP) and a mature TGFB1 peptide, and is found in either a latent form composed of a TGFB1 homodimer, a LAP homodimer, and a latent TGFB1-binding protein, or in an active form composed of a TGFB1 homodimer. The mature

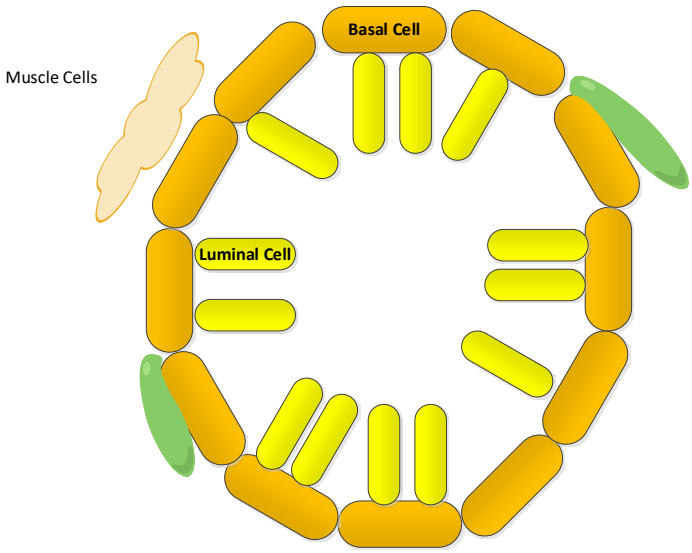
[16] See White Paper No. 133 LY6 and Prognostic Markers (February 2016)

peptide may also form heterodimers with other TGFB family members. This gene is frequently upregulated in tumor cells, and mutations in this gene result in Camurati-Engelmann disease.

We now focus on the issue of prostate cancer, PCa, and its stem cell as well as its cancer cell of origin, CSC and CCO.

The prostate is an interesting organ. It is a collection of glandular cell segments and the glands contain a circumference of basal cells and a collection of luminal cells extending into the lumen, the empty space. Around the periphery and in the connective space are a multiplicity of other cells of various types; muscle cells, neuroendocrine cells and the like. The prostate tends to grow or enlarge as a man gets older and thus can grow from a typical size of 40 cc to at times well over 100 cc. In simple age related benign growth the prostate duplicates itself in an ever enlarging glandular network and generally appears somewhat uniform. Inflammation may occur as well as hyperplasia.

The hyperplasia generally appears as masses of excess and somewhat disordered luminal cells in the lumen, and the organization of the lumen begins to become distorted. In the extreme case of High Grade Prostatic Intraepithelial Hyperplasia, HGPIN, the gland appears almost filled with luminal cells. Some have argued that this is a precancerous state and irreversible. We however have seen cases where it is totally reversible and thus this existence proof of non-inevitability is questionable.



As Agarwal et al note:

Prostate glands are composed of:

1. *an outer layer of basal cells expressing KRT5, KRT14, and TP63,*
2. *an inner layer of secretory, luminal cells expressing KRT8, KRT18, and AR,*
3. *and rare SYP and CHGA positive neuroendocrine cells.*

TP63 is a marker of prostate basal epithelial and stem cells and is required for prostate development. Lineage tracing studies based upon cytokeratin drivers have established a number of principles for stem cell hierarchies in the developing and adult prostate.

The majority of regenerative adult stem cells appear to be unipotent. In addition, studies using other lineage tracing schemes have described minor populations of multipotent progenitor cells that have not been captured with KRT-specific drivers.

Using an inducible NKX3.1-specific CRE driver, a rare (0.7%) population of bipotential luminal cells in the castrate prostate (CARNs) has been described (Wang et al., 2009). In addition, the existence of KRT5neg, KRT14-,TP63+ cells has been observed, as well as the ability of TP63 lineage marked cells to generate luminal epithelial cells in the adult

As PCa develops it initially appears as a multiplicity of poorly formed glandular structures, and although looking somewhat like the benign normal glands it starts to loose structure as it develops. The question than is; what cells is the basis for this change, basal or luminal or other, and as the PCa starts to expand which cell is and/or becomes a CSC?

From White and Lowry, we have a summary of the issue regarding the cell of origin for PCa. They note:

Models for both murine and human prostate cancers have produced conflicting conclusions within the field as to whether the CCO is of basal or luminal origin. Debate has arisen as to whether the stem cells of the prostate reside in either the basal or luminal populations.

Using a broader range of lineage tracing alleles, it was suggested that a multipotent population arises from the basal population, while separate unipotent progenitors populate the neuroendocrine and luminal pools. The lack of a consensus on the identity of ASCs of the prostate has also clouded the interpretation of CCO studies for the prostate. Similar to the discrepancies observed for SCC/BCC, much of the debate regarding CCOs for prostate cancer centers on the fact that prostate tumors typically adopt a morphology consistent with a luminal origin, while experimental data often point towards a basal source for CCOs.

Human prostatic epithelial transplantation studies, which do not include a native stromal and immune component, indicated a basal CCO with MYC, AKT or ERG as oncogenic drivers. By contrast, genetically modified mouse models that used Pten deletion implicated both basal and luminal cells as CCOs, depending on the targeting alleles and tumorigenic strategies used.

In addition, one study showed that initiation from human basal cells generates transformed luminal-like cells that are able to propagate the tumor. Together, these results suggest that the identity of the CCO for prostate cancer could be dependent on cellular, genetic, and environmental contexts, and further work will be needed to address whether differences exist between human and mouse models systems or whether the differences are caused by nonequivalent cell-intrinsic and cell-extrinsic stimuli.

Heterogeneity of tumor initiators and tumor phenotypes The experimental models described here have proven to yield important insights into tumor initiation and CCOs. However, there are technical limitations to these models that ignore the heterogeneity of bona fide cancer initiation. Tumors are thought to be initiated in a clonal fashion as a result of mutations.

We thus examine this CCO and corresponding CSC issue for PCa.

The Cell of Origin is akin to the CSC and has been the focus of debate in PCa. As White and Lowry have noted:

Significant progress has been made to identify the cells at the foundation of tumorigenesis, the ***cancer cell of origin (CCO).*** *The majority of data points towards resident* ***adult stem cells (ASCs) or primitive progenitors as the CCO for those cancers studied, highlighting the importance of stem cells not only as propagators but also as initiators of cancer****. Recent data suggest tumor initiation at the CCOs can be regulated through both intrinsic and extrinsic signals and that the identity of the CCOs and their propensity to initiate tumorigenesis is context dependent. In this review, we summarize some of the recent findings regarding CCOs and solid tumor initiation and highlight its relation with bona fide human cancer.*

Cancer is a complex disease due to the wide variety of cellular and molecular mechanisms associated with its initiation and progression. It is accepted that cancer cells divide and proliferate uncontrollably because of the accumulation of somatic mutations in normal tissue, which confers a selective growth advantage in the mutated progeny.

However, the cells that make up a tumor are heterogeneous; often making it difficult to determine the ***CCO, which is the normal cell that acquires the mutational load necessary to first initiate cancerous proliferation****. Furthermore, since cancer is a transformative process, the cells composing advanced cancers may no longer contain morphological or molecular characteristics of the CCO. The identity of the CCO could be critical to the generation of more effective treatments and preventative strategies.*

If CCOs can be identified and targeted specifically, it would be possible to stop cancer before it has a chance to undergo expansion. Molecular or physiological attributes specific to CCOs could be exploited to slow or block progression, thus avoiding treatments that simply kill dividing cells. This has led to significant recent efforts to define CCOs for all types of cancers, and numerous lines of evidence point towards ASCs as possible CCOs.

They continue:

ASCs are found in many of the major adult organs and are essential for tissue homeostasis as well as regeneration in response to injury*.*

Most ASCs were discovered on the basis of their relative quiescence and their ability to reconstitute differentiated cell lineages of the tissue or organ in which they. Either upon activation by natural turnover/cycling or in the case of regeneration due to injury, ***ASCs give***

rise to multilineage restricted progenitors or, as they are often called, transit amplifying cells (TACs).

These cells divide rapidly and then differentiate to generate the bulk of cells required for tissue turnover or regeneration. Due to their rapid division, TACs are also targeted by chemotherapeutics that act on cell division pathways to kill cancer cells

In the above we have seen defined three entities:

1. CCO: The cancer cell of origin.

2. ASC: Adult stem cells.

3. TAC: Transit amplifying cells

Wang and Shen note:

A similar confusion arises with respect to the cell of origin for cancer, which corresponds to a normal tissue cell that is the target for the initiating events of tumorigenesis. In principle, a normal adult stem cell could be a logical cell of origin for cancer, as it would retain the ability to self-renew and generate a hierarchy of differentiated lineages within a tumor. However, it is also possible that a cell of origin could correspond to a downstream progenitor cell or conceivably even a terminally differentiated cell that acquires stem cell properties during oncogenic transformation.

Thus we even here have some confusion as to the CCO, cancer cell of origin.

One of the sets of arguments presents the basal cell as the cell of origin. As Wang and Shen note:

Although prostate tumors display a strongly luminal phenotype, this does not exclude the possibility that basal cells could be a cell of origin for prostate cancer. In particular, it is possible that transformed basal cells could differentiate to generate large numbers of luminal cancer cells. For example, prostate-specific conditional deletion of Pten by a probasin-Cre driver allele has been shown to result in a basal cell expansion accompanied by increased number of intermediate cells, suggesting a basal cell of origin.

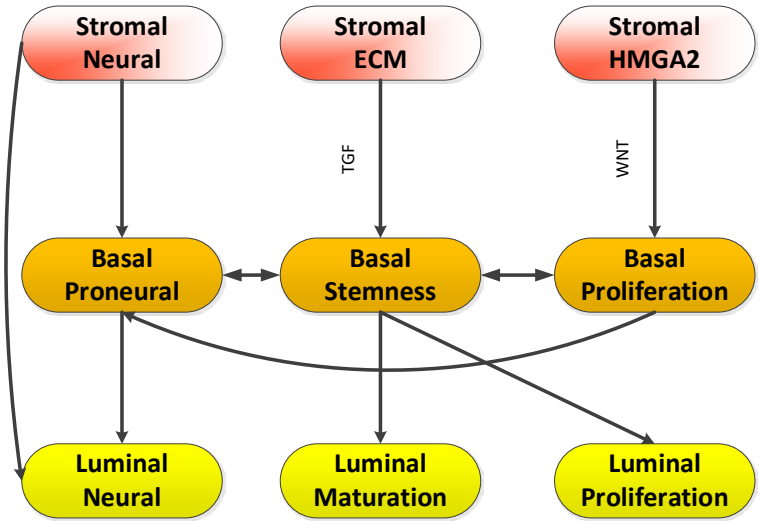
In a paper by Goldstein et al they note:

Luminal cells are believed to be the cells of origin for human prostate cancer, because the disease is characterized by luminal cell expansion and the absence of basal cells. Yet functional studies addressing the origin of human prostate cancer have not previously been reported because of a lack of relevant in vivo human models. Here we show that basal cells from primary benign human prostate tissue can initiate prostate cancer in immunodeficient mice.

The cooperative effects of AKT, ERG, and androgen receptor in basal cells recapitulated the histological and molecular features of human prostate cancer, with loss of basal cells and

expansion of luminal cells expressing prostate-specific antigen and alpha-methylacyl-CoA racemase. Our results demonstrate that histological characterization of cancers does not necessarily correlate with the cellular origins of the disease.

We had examined this in some detail when it first appeared some six years ago. The problems were that it was murine related and one could argue that CSC in a mouse is not CSC in human. Suggestive but not a definitive proof.



From Moscatelli and Wilson we have the arguments:

In a recent paper in Science (3), Goldstein et al. describe a model system in which questions about the cell of origin and oncogenic pathways of human prostate cancers can be addressed. Using two cell surface antigens, Trop2 (TACSTD2) and CD49f (integrin α6), Goldstein et al. (3) separated luminal (Trop2+/CD49f−) from basal (Trop2+/ CD49f+) cells in digests of benign human prostate tissue.

When each of these populations, along with urogenital sinus mesenchyme cells that promote the proliferation of primitive prostate cells, was injected subcutaneously into immunodeficient (NODSCID- IL2Rγ−/−) mice, the basal cell population gave rise to prostate-like structures containing both basal and luminal cells, whereas the luminal population did not grow, confirming observations from mouse prostate (4) that the basal layer contains prostatic epithelial stem cells.

Goldstein et al. (3) then used lentiviral vectors to transform these cells with genes encoding activated Akt and ERG, which are commonly associated with human prostate cancers. When transplanted into the mouse, the transformed basal cells formed tissues that resembled prostatic intraepithelial neoplasia (PIN) (that is, microscopic groups of atypical epithelial cells that represent a premalignant state), containing both basal and luminal cells, whereas transformed luminal cells did not grow.

Finally, addition of the androgen receptor gene, which is often up-regulated in prostate cancer, to the genes expressing activated Akt and ERG in the basal cells gave rise to frank adenocarcinomas with an expanded luminal cell population and an absence of basal cells, whereas expression of these same genes in luminal cells did not generate any prostatic tissue.

The authors conclude that basal stem cells are the target of transformation in the generation of prostate tumors.

Finally, in the recent study by Zhang et al they conclude:

The current study has made the following significant findings (see Supplementary Discussion).

First, our study uncovers unique SC- and EMT-enriched gene-expression profile in unperturbed basal cells that support the long-held hypothesis that the human prostate basal cell layer harbors primitive SCs.

Second, we report the surprising finding that basal cells are enriched in genes normally associated with neurogenesis. In contrast, luminal cells preferentially express proneural genes involved in neural signal response and processing. Consistently, primary basal cells can spontaneously or be induced to undergo 'neural' development in vitro, generating NSC-like cells. Combined with the SC features, these transcriptional programs provide a molecular understanding for the reported basal cell plasticity.

Third, basal cells express high levels of Pol I-associated rRNA biogenesis genes regulated, at least in part, by the MYC transcriptional programme. MYC is often found overexpressed in PCa, especially metastatic PCa. Increased transcription of rRNA genes by Pol I is a common feature of human cancer. Thus, our data may suggest a rationale for treating anaplastic PCa and CRPC with Pol I inhibition, as well as targeting MYC and the MYC-mediated transcriptional programme as a therapy for PCa.

Fourth, our deep RNA-Seq data provide a rich resource for epithelial lineage specific genes and markers in the human prostate.

Fifth, distinct transcriptomes in basal and luminal cells also suggest cross communications between the two epithelial cell types, as well as between the epithelial compartment and the underlying stroma. Understanding such crosstalk will be instrumental for understanding the normal development and tumorigenesis of prostate. Although many of the signaling pathways mentioned in this study are poorly investigated in normal prostate epithelial biology, their functional involvement in PCa development and progression has been widely documented3.

Last, the basal cell gene-expression profile is linked to adverse clinical features of PCa, indicating a 'biomarker' value of basal cell gene signature for aggressive PCa.

Importantly, the molecular resemblance of basal cells to anaplastic PCa and CRPC provides a common molecular understanding of these diverse and poorly characterized aggressive PCa subtypes and implicates basal cells as the cell-of-origin for these variant PCa. It should be noted

that while this manuscript was under review, another paper reported similar findings in linking the basal cell gene expression to aggressive PCa

The above by Zhang et all appears to be the most comprehensive argument for CCO as basal.

There is a set of counter proposals for the luminal cells as the CCO.

Specifically, another set of arguments defends the luminal cell, namely Wang and Shen have noted:

Other studies have provided evidence that luminal cells can serve as cells of origin for prostate cancer. For example, pathological analysis of high-grade PIN samples, which still retain basal cells, suggest that molecular events associated with human prostate cancer initiation such as upregulation of c-MYC and shortening of telomere length occur exclusively in luminal cells but not their basal neighbors. In mouse models, a recent study using a prostate-specific antigen-Cre, PtenloxP/loxP prostate cancer model reported that the initial hyperplastic cells were all luminal. Finally, our laboratory has shown that targeted deletion of Pten in CARNs resulted in high-grade PIN and carcinoma, indicating that CARNs are a cell of origin. At present, however, it is unknown whether CARNs exist in the hormonally intact prostate epithelium, and if so, whether these cells can serve as cells of origin. Indeed, if CARNs correspond to facultative stem cells, as discussed above, they may correspond to a cell state that is only acquired in the regressed epithelium.

Also from Moscatelli and Wilson we have the arguments:

At first glance, these findings seem to be in conflict with those in a recent paper from Wang et al. (5) that concludes that a luminal epithelial stem cell is the target of transformation in prostate cancer. This conclusion relies on lineage-tracing studies in the mouse prostate. Wang et al. (5) found that expression of a prostate-specific homeobox gene, Nkx3-1, marked rare luminal epithelial cells but was never observed in basal cells in prostates after castration-induced involution. When mice are castrated to abolish the production of testicular androgens, the prostate involutes, resulting in a reduction in size due to apoptosis of most luminal cells and of a small fraction of basal cells.

When androgens are readministered, the prostate regenerates. When castration-resistant Nkx3-1–expressing cells (CARNs) marked with yellow fluorescent protein (YFP) were followed, it was found that these cells expanded over ninefold during regeneration of the prostate after androgen replenishment and gave rise to luminal, basal, and neuroendocrine cells.

Reimplantation of single YFP-marked CARNs, along with urogenital sinus mesenchyme, under the renal capsule (a fibrous layer surrounding the kidney) of immunodeficient (nude) mice generated prostatic ducts containing both basal and luminal cells that were completely YFP positive.

Specifically deleting the tumor suppressor gene PTEN (which regulates the Akt signaling pathway and is often inactivated in human prostate cancer) in CARN cells led to the rapid

development of tumors with a luminal phenotype and an absence of basal cells upon prostate regeneration. These results suggested that CARNs are prostate stem/progenitor cells and targets of transformation.

Similarly, in a recent (2015) paper by Agarwal et al the authors note:

Primary prostate cancer almost always has a luminal phenotype. However, little is known about the stem/ progenitor properties of transformed cells within tumors. Using the aggressive Pten/Tp53-null mouse model of prostate cancer, we show that two classes of luminal progenitors exist within a tumor. Not only did tumors contain previously described multipotent progenitors, but also a major population of committed luminal progenitors.

Luminal cells, sorted directly from tumors or grown as organoids, initiated tumors of adenocarcinoma or multilineage histological phenotypes, which is consistent with luminal and multipotent differentiation potentials, respectively.

Moreover, using organoids we show that the ability of luminal-committed progenitors to self-renew is a tumor-specific property, absent in benign luminal cells.

Finally, a significant fraction of luminal progenitors survived in vivo castration. In all, these data reveal two luminal tumor populations with different stem/progenitor cell capacities, providing insight into prostate cancer cells that initiate tumors and can influence treatment response.

Thus using this model, we again see an argument for luminal cells.

4.7 OBSERVATIONS

The various sides of the arguments presented herein most likely continue. As much as murine models have value they also are a substantially different species.

4.7.1 How Close is Close?

The issue of how close we should be examining the tumors is a critical one. As Gundem et al have noted the PCa tumors are very genetically heterogeneous. In the development of a metastatic state the original tumor spreads and optimizes itself to the environment in which it is best suited. Thus as is frequently the case the PCa tumors seek presence in the bone and restructure the bone in their own liking. The question is then; what are the spatio temporal changes we see and can they become elements of therapeutic targets?

To understand this better, we again examine the literature. In the conclusion to the Navin and Hicks paper they state:

Biological models are by definition built upon incomplete information. At best, these explicit models for tumor progression provide guideposts for further exploration. As technology

continues to evolve, the analysis of cancer samples of complex mixtures will give way to methods aimed at the individual cell.

Such methods will enable single cancer cells to be tracked as they progress to form the primary tumor and traced as they migrate through the body to seed the metastasis. In the near future the cost of deep sequencing a mammalian genome, whether from a tumor sample or a few disseminated cells will be approximately equivalent to the current price of a microarray experiment. Single cell genomes are also ideal for constructing detailed lineages of tumor progression, because individual mutations in a genome can be traced as they are inherited and expanded in subpopulations.

As we bring the magnifying glass closer, we may also be able to track the genetic stepping stones for tumor growth, or follow the genetic changes in circulating tumor cells as they progress from the primary to metastasis. Perhaps, we will find evidence that individual circulating tumor cells return to the primary tumor after developing offsite as the self-seeding model suggests. It is then that these predictive genetic models will have *realized their full value.*

It is reasonable to consider that examining the cell by cell profile of a cancer will be exceptionally enlightening. In addition to understand from the tumor progression how the malignancy changes in time and place is also critical. The issues as to what causes a cell to proliferate and mutate is essential to understanding how to target the cell. Perhaps if the CSC model is correct and that if we target the CSC itself then the other cells just die off.

4.7.2 Cell Import

What cell should we focus on and how do we identify it? As much as we have gathered about PCa and its genetics, we are still often in the dark because we lack the equivalent of the simplicity of a set of Newton's Laws. The state of a PCa cell is stochastic and does not follow the ballistic parabolic flight of a Newtonian projectile. Thus "Moon Shots" are problematic at best. We may still be hurling stones from Roman like launches.

From Agarwal et al:

This study characterizes primary prostate tumors initiated by loss of the common tumor suppressors, Pten and Tp53, for stem/progenitor phenotypes as assayed by in vitro organoid cultures and in vivo tumor-initiating activity.

It has not been routinely possible to culture luminal stem/progenitor cells, which has prevented ex vivo analysis of these important cells in primary prostate tumors, biasing most studies toward primary basal cells or human prostate cancer cell lines.

We have observed two classes of self-renewing luminal progenitors in Pten/Tp53-null tumors, a minor population giving rise to multilineage organoids (multipotent progenitors) and a major population producing luminal-only organoids (luminal committed progenitors). Of particular interest is the observation that multilineage organoids give rise to self-renewing luminal

organoids, providing additional insight into progenitor subpopulations, lineage stages leading to luminal commitment, and one route of prostate adenocarcinoma mitogenesis.

We suggest that combined loss of Pten and Tp53 either in the luminal multipotent progenitor or a precursor has revealed a naturally transient population, possibly by inhibiting the normal rate of differentiation. This interpretation is consistent with considerable evidence linking Tp53 to the regulation of differentiation in stem cells.

To date, luminal multipotent progenitor cells have not been observed in lineage tracing experiments, except in the case of rare CARN's, prompting questions about the significance of the multipotent progenitors revealed in organoid cultures. We show the existence of multipotent and luminal-committed TICs isolated directly from tumors, producing either adenosquamous carcinoma or adenocarcinoma, respectively. Importantly, the TIC assays used here measured autonomous differentiation potential in the absence of inductive embryonic urogenital mesenchyme. Endogenous adenosquamous prostate carcinoma is observed in a fraction of PB-CRE4; Ptenfl/fl;Tp53fl/fl mice, supporting the concept that transformed multipotent progenitors exist in vivo and can differentiate to both basal and luminal lineages in tumors in situ.

It seems likely that the microenvironment will influence lineage commitment, and we note that organoids and TIC assays are performed in the absence of stromal cells. Therefore, it is possible in these assays that the extent of basal cell commitment by multilineage progenitors may be increased relative to the endogenous microenvironment.

Although engineered models of prostate cancer are often used to analyze the consequences of combined genetic mutations, the effect upon stem/progenitor populations has not been commonly considered. We show here for PB-CRE4-initiated genetic changes that Tp53 in combination with Pten loss demonstrated significantly different stem/ progenitor populations compared to Pten loss alone.

Specifically, Tp53 loss leads to the presence of luminal multipotent stem/progenitor cells and a self-renewing luminal population, correlated with accelerated adenocarcinoma development, that is absent in Pten-null prostates. In addition, it is possible that Tp53 loss primes for lineage plasticity, similarly to the phenotypic dedifferentiation of luminal mammary epithelium following Brca1 loss. Analyses of stem/progenitor populations contribute fundamental knowledge for molecular and pathological comparisons of GEM models and for interpretation of target populations responding to therapeutics…

Due to a lack of biomarkers, the extent of innate stem/progenitor subpopulation heterogeneity in human prostate cancer is not known.

The metaphor of launching stones is apropos. We cannot truly identify the targets and we do not have the predictive tools of Newton.

4.7.3 Alternative Views

It appears that most if not all of the work on understanding the cancer dynamics has been from the cell upwards[17]. The CSC has become a focal point, and paradigm for the bench work from which possibly prognostic, diagnostic and therapeutic approaches could evolve. We have on the other hand examined the process from the top down. Namely we looked at the gross characteristics of cumulative collections of common cell states. We have defined a metric which is the local cell density of a cell having a specific genetic state, which may also include a specific epigenetic state as well. Namely we define:

$$\overline{n}_j(x,t) = E[n_j(x,t)]$$

Where j is a genetic state which may be for example:

$$j = \{all \text{ cells such that genes k=1,...,N are functional and genes belonging to the set } \Omega(j) \text{ are present}\}$$

$$\Omega(j) = \{\text{set of all genes when gene } G_j \text{ is aberrant, ie BRAF V600}\}$$

Thus if we admit a total of J states and we admit that states can transition and that each state has a growth mechanism as well as a flow and diffusion mechanism then we can determine the spatio-temporal values for the average densities simply by solving:

$$\frac{\partial \overline{n(x,t)}}{\partial t} = \widetilde{L}\overline{n(x,t)} + \Lambda \overline{n(x,t)}$$

where

$$\widetilde{L} = \left[\widetilde{L}_1 ..... \widetilde{L_N}\right]$$

and

$$\Lambda = \begin{pmatrix} -\lambda_{11} & \lambda_{12} & \lambda_{13} \\ \lambda_{21} & -\lambda_{22} & \lambda_{23} \\ \lambda_{31} & \lambda_{32} & -\lambda_{33} \end{pmatrix}$$

Where the L values are operators reflecting diffusion and flow while Λ is a growth related value. We assume that states transition with certain probabilities, which can be ascertained phenomenologically. Thus we have:

[17] One should examine the work by Tan et al. Although they do not pose the problem as we have they do start examining the work from the perspective of a state space with spatial and temporal complexity. Regrettably their focus is on the mathematics and not the phenomenology.

Let

$$p(i,x,t) = P[n(x,t) \in S_i] = p_i(x,t)$$

$$S_i = Gene\ State(i)$$

Assume

$$p_j(x,t+\Delta) = P_{j,i}\, p_i(x,t)$$

and

$$P_{j,i} = \begin{cases} P_{i,i}(1-\Delta) = \\ \dots \\ P_{j,i}\Delta = \lambda_{i,j} \\ \dots \end{cases}$$

And we can conclude:

$$P = \begin{bmatrix} p_{1,1}..p_{2,1}..p_{2,2}..p_{2,3}..0.....0.....0.....0 \\ 0.....p_{2,2}..p_{2,5}..p_{2,6}..p_{2.7}..0.....0.....0 \\ 0.....0.....p_{3,3}..p_{3,5}..p_{3,6}..p_{3,7}..0.....0 \\ 0.....0....0.....p_{4,4}..p_{4,5}..p_{4,6}..p_{4,7}.....0 \\ 0.....0....0.....0.....p_{5,5}..0.....0.....p_{5,8} \\ 0.....0....0.....0.....0.....p_{6,8}.....0.....p_{6,8} \\ 0.....0....0.....0.....0.....0.....p_{7,8}....p_{7,8} \\ 0.....0....0.....0.....0.....0.....0.....p_{8,8} \end{bmatrix}$$

Is the totality of these transitions.

We have demonstrated how one may actually estimate or identify the value of these gross parameters for any cancer. The recent work of Gundem et al has shown also how this works. We have further demonstrated an example of this for PCa.

In a recent paper by Smith et al the authors have a model somewhat akin to what we presented several years before. Namely they state:

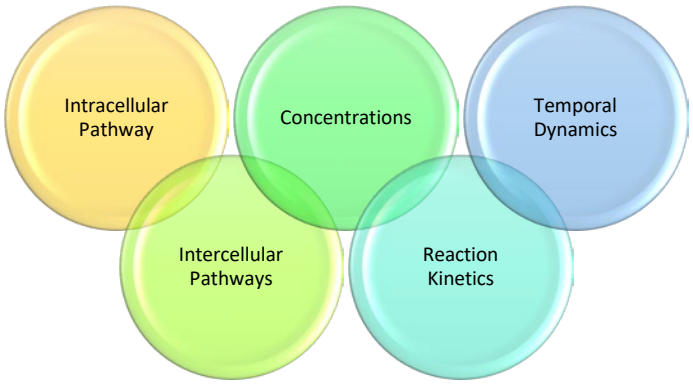
By analyzing stem cell differentiation dynamics in many spatially defined microenvironments, we found strong stochastic behavior during the differentiation process. The composition of individual micropatterns varied dramatically over the time course of the differentiation. On smaller micropatterns, we observe that the most probable composition is either 100% stem cells or 100% differentiated cells.

Moreover, the physical dimensions of the microenvironment can influence stem cell differentiation in significant ways. We propose a stochastic differentiation model frame-work, and showed that stem cell differentiation probability is a strong function of local stem cell fraction within the immediate cell vicinity.

When stem cells are surrounded by other stem cells, the differentiation decision is slow; whereas, when differentiated cells surround stem cells, then the differentiation rate is faster by nearly threefold. This result is consistent with the previous proposal that there are feedback signals between differentiated cells and stem cells16. The proposed stochastic modeling framework should be applicable in other settings for understanding differentiation dynamics. We also found that the cell-cell interaction during differentiation is partially mediated by an E-cadherin governed signaling mechanism. Although, cell-cell interaction is not completely inhibited in our experimental conditions, we are able to manipulate, observe, and quantify variances in differentiation kinetics when the roles of cell contact in spatially confined domains are altered.

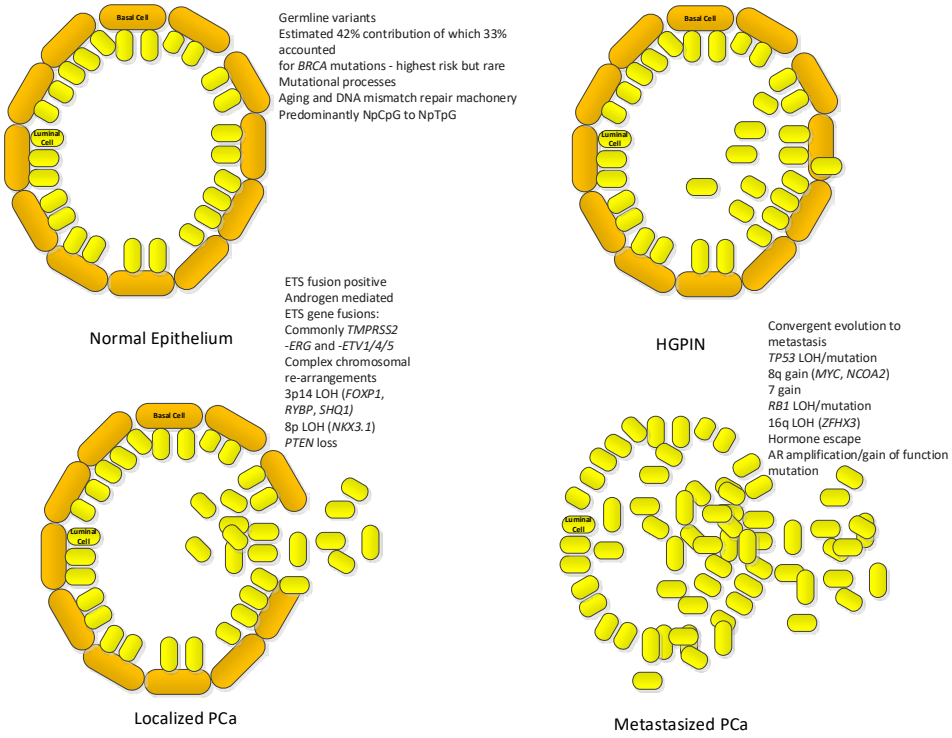
It is possible that E-cadherin affects multiple sensing mechanisms in stem cells and there are redundant mechanisms that reinforce cell-cell interaction in stem cell niches.

We have demonstrated a model containing the key elements shown below.



In a sense this is also what Smith et al are trying to develop. We believe that by examining the cancer in a large scale stochastic manner we can utilize current knowledge and develop new understanding. The cancer in our model is considered almost as a separate entity existing in a human body, and it uses the characteristics of its carrier, the human, to facilitate its growth. The human is in homeostasis and the cancer entity is competing with the human for resources to survive and prosper.

Considerable understanding on the details of PCa cell complexity has become available recently (see Gundem et al and Mitchell and Neal) From Mitchell and Neal we have the following Figure:



The question then is: in this phenomenological complex, what is the role of the CCO and CSC? One can consider the gene expression changes, due to mutations, epigenetic factors or otherwise, then combined with ligands that prompt pathways to operate and for the gene expression changed cell to proliferate and/or produce other growth factors and/or impact the extracellular matrix changing adhesion to see this new "organism", the tumor mass, to spread and alter itself to maximize its growth potential.

In essence we have a Darwinian sub-process allowing this new "organism" to prosper. To counter this process, we must identify the control mechanisms, all, not just a few, and then suppress them.

5 TOOLS

Immunotherapy as well as all of the current progress in understanding and manipulating cells, DNA, and the immune system are built upon a large base of "tools". These tools are manipulative devices that can cut, replace, change, repair, modify DNA, RNA, proteins and the like. We briefly examine these tools.

It should be stated that these "tools" are but a few which can be used as well as the fact that new tools are being introduced continuously. Tools provide a means whereby one can modify cells to accomplish tasks which the immune system can normally perform, but that these tasks are now performed to target specific aberrant cells.

5.1 Some Key Tools

Specifically, we look at:

1. CRISPR: The CRISPR tool set uses a technique found in bacteria wherein an endonuclease, often Cas9, is used to cut DNA and a targeted RNA strand, the CRISPR, used to select where to cut. Thus, one may obtain a cut and paste. Some applications to cancer therapy have been developed. However, it is reasonable to assume that using this tool one may alter immune system cells to target specific cancers.

2. Mab: Monoclonal Antibodies, Mabs, are the production of antigen, Ag, specific antibodies. Antibodies are as we have discussed, protein structures which naturally form in the immune system and when activated can become targeting mechanisms for the attack on antigen presenting pathogens or cells containing such. They also can target any identifiable epitope or region to which they can be attached. Thus, on the one hand we can use Abs to attack specific cells and on the other hand we can use Abs to inhibit the activation of cells by attaching to receptors and inhibiting activating ligands. The logic of how Abs can be employed demonstrates how we can take a naturally occurring process and modify and use it for disease inhibition.

3. Gene Drives: The gene drive is a mechanism which works in embryonic type cells, such as stem cells. Namely we place a desired gene into a cell along with a CRISPR and then when the cells multiply we are assured of dual gene functioning, namely all genes have and express the same set of genes. Perhaps this tool will allow for the elimination of such diseases.

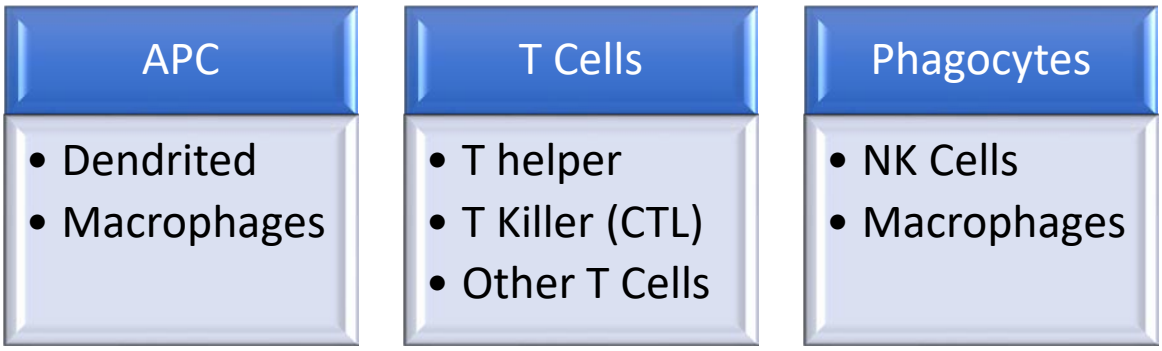
4. CAR T: These are techniques where we can add a chimeric portion to a receptor or ligand and it has demonstrated efficacy in many hematological cancers.

Each of these is somewhat unique. They do not in any way represent the totality of tools but they do provide a window of what can be made available to cell manipulation.

5.2 Strategies to Apply

There can be a wide variety of applications. They can be by cell type, cell function, antibody type, and the like.

By cell function we could have the following selection of such cells. This range of cells goes from those which seek out and present antigens, to those which are immediate responders to those which are inherent parts of the adaptive immune system.



In the context of the immune system we have cells which:

1. Identify antigens or molecules which should not be there
2. Cells which can take up those antigens and communicate them to other parts of the immune system.
3. Receptors which are activating with respect to the immune system.
4. Receptors which are inhibitors of the immune system.
5. Ligands which can bind to any of the above receptors.
6. Molecules which enhance the action of the immune cells.
7. Cells which can attack and kill off invading cells as a part of the innate system.
8. Cells which can adapt to the presence of a new antigen and then target specifically that antigen on a cell.
9. Assisting cells that enhance the immune system.

The challenge is to understand what these are as to understand the "natural" mechanism by which the work, and then to understand how a cancer cell evades that natural mechanism. Cancer cells have the advantage of being part of the normal cell line, namely that is from whence they came.

Yet the cancer cell seems to be able to act differently. The Immune System can somewhat easily identify alien cells bit not so well self-cells which are functioning in an alien manner. The challenge of Immunotherapeutics to cancer is to somehow enable the immune system to recognize cells which do not behave normally and to eliminate them.

5.3 Focus of Presentation

The focus on the following sections is to present some tools, and tools which are significant enough to present the opportunity to effect major changes in the immune system's ability to deal with cancer cells.

6 CRISPR

CRISPRs, specifically "clustered regularly interspaced short palindromic repeats"[18], are portions of a cell's DNA which contain a particular type of short repetitions.. These specific repetitions are then followed by additional short segments of DNA which have been collected from some prior exposure to a virus phage. Namely CRISPRS are selective DNA snippets which have been garnered from viral phages which in some past period tried to attack the prior lineage of this cell. They are used to create Cas ("CRISPR associated" genes) which in turn have the capability of cleaving genes and inserting new ones.

CRISPR-Cas systems are now a useful toolkit for engineering eukaryotic cells, and especially human cells. They are also used in plant cells and that is a second tale but one worth examining as well.

As Jinek et al have recently said (Jinek et al 2014):

Although type I and III CRISPR-Cas systems rely on multiprotein complexes for crRNA-guided DNA targeting, type II systems use a single RNA-guided endonuclease, Cas9, that requires both a mature crRNA and a trans-activating crRNA (tracrRNA) for target DNA recognition and cleavage (8, 9). Both a seed sequence in the crRNA and conserved protospacer adjacent motif (PAM) sequence in the target are crucial for Cas9-mediated cleavage.

The use of the crRNA and the tracrRNA are the two key elements which we shall discuss in this process. Also the Type II CRISPR-Cas system is the one which has received the most attention.

Cas9 proteins are abundant across the bacterial kingdom, but vary widely in both sequence and size. All known Cas9 enzymes contain an HNH domain that cleaves the DNA strand complementary to the guide RNA sequence (target strand), and a RuvC nuclease domain required for cleaving the noncomplementary strand (nontarget strand), yielding double-strand DNA breaks (DSBs).

These DSB open up the DNA at a desired location. Thus if one has a specific gene to be spliced out, and to be replaced, the first step is to open the DNA at the site of that desired gene. Thus the above step is a critical first step.

[18] Recall that a palindrome is a collection of letters which can be read the same forwards of backwards. For example; GCATTACG.

In addition, Cas9 enzymes contain a highly conserved arginine-rich (Arg-rich) region previously suggested to mediate nucleic acid binding. On the basis of CRISPR-Cas locus architecture and protein sequence phylogeny, Cas9 genes cluster into three subfamilies: types II-A, II-B, and II-C. Cas9 proteins found in II-A and II-C subfamilies typically contain ~1400 and ~1100 amino acids, respectively.

The ability to program Cas9 for DNA cleavage at specific sites defined by guide RNAs has led to its adoption as a versatile platform for genome engineering . When directed to target loci in eukaryotes by either dual ***crRNA:tracrRNA*** *guides or* ***chimeric single-guide RNAs****, Cas9 generates site-specific DSBs that are repaired either by nonhomologous end joining or by homologous recombination, which can be exploited to modify genomic sequences in the vicinity of the Cas9-generated DSBs.*

The opened DNA then can be targeted by crRNA:tracrRNA segments that remove and replace the targeted DNA or by a chimeric single-guide RNA which accomplishes this all in one step. This is the second step in CRISPR gene targeting and re-engineering. We shall discuss this a bit more later.

Furthermore, catalytically inactive Cas9 alone or fused to transcriptional activation or repression domains can be used to control transcription at sites defined by guide RNAs. Both type II-A and type II-C Cas9 proteins have been used in eukaryotic genome editing. Smaller Cas9 proteins, encoded by more compact genes, are potentially advantageous for cellular delivery using vectors that have limited size such as adeno-associated virus and lentivirus.

CRISPR, those collections of small sets of palindromic DNA inserted in the hosts original DNA, can be collectively called a process that is naturally occurring in nature and it is also a procedure that can then be implemented across a wide selection of cell types. In a sense it has been called the lower organism's immune system, a means of remembering previous attackers to the organisms such as bacteria, and a way to use that memory as a defense mechanism against future attacks. The mechanism can then be used in higher level organisms as a reverse process, a means of attacking bad genes and then inactivating them. It is in effect a trick to take what lower organisms have developed for protection and employ in higher level organisms for therapeutic purposes.

In a recent paper by Villion and Moineau the authors examine the two sides of CRISPR, the side that adds segments of foreign DNA to enable an immune type system and the side that deletes selected DNA.

To cope with this never-ending threat, microorganisms have developed a wide range of defense mechanisms.

Among them, CRISPR-Cas system is the new kid on the block as its silencing role was reported only five years ago. An outburst of articles, meetings, and reviews has since followed, arguably making it one of the hottest topics in microbiology.

CRISPR (clustered regulatory interspaced short palindromic repeats) loci are found in approximately 45% of sequenced bacterial genomes as well as 90% of archaeal ones and one genome can contain multiple CRISPR loci. Variable short regions, called spacers, separate each of the short repeats. The spacers are mainly homologous to viral or plasmid sequences. CRISPR-associated (cas) genes are often located adjacent to the CRISPR locus. The diversity and specificity of the cas operons has led to the identification of signature cas genes and to a polythetic classification scheme for CRISPR-Cas systems (types I to III, with several subtypes).

Notwithstanding their particularities, CRISPR-Cas systems operate through three general steps to provide immunity. In the adaptation stage, some cells will respond to the invasion of a phage or a plasmid by adding a new repeat-spacer unit into the CRISPR array, mostly polarized at the 5′ end. Strikingly, the spacer sequence comes from the invading nucleic acid while the newly added repeat derives from another repeat of the array.

The mechanistic details on how this adaptation/immunization occurs are still unknown but some Cas proteins are involved. The unique spacer content is now considered a sign of past challenges and can serve as a marker for strain typing. In the second step, small non-coding CRISPR RNAs (crRNAs) are generated. A long precursor CRISPR RNA is first produced from an AT-rich leader/promoter region, which is then processed within the repeats and mature into crRNAs.

Several Cas proteins participate into the biogenesis of crRNAs. Finally, in the interference stage, the crRNACas protein complex will bind to the invading nucleic acid target and cleave it, providing a defense system to the host microbe. Therefore, CRISPRCas systems are RNA-based adaptive microbial immune systems that target nucleic acid intruders.

They end with the following, the double edged sword portion:

Although already outstanding in bridging gaps in our understanding of CRISPR-Cas systems, this fascinating story does not end here. The authors investigated the possibility of using this dual-RNA system to program Cas9 to specifically cleave any desired DNA molecules. Minimal requirements to have an efficient single chimeric RNA molecule mimicking the dual RNA structure were defined and led to site specific DNA cleavage by Cas9. In fact, several different chimeric guide RNAs were engineered and used to cleave a plasmid containing the specific target and a PAM. These findings coupled to the previous observations that CRISPRCas systems can be functionally transferred from one organism to another open up exciting possibilities for gene targeting and genome editing of microbes and even higher organisms.

That is they have developed a way to reverse the process, using the mechanism now, not to add a piece of DNA, but to cleave a piece of DNA. This opens the door for many types of treatment of cancers where we may know the genetic defect and then can cut it out, cell by cell.

We examine briefly herein some of the recently discovered uses of CRISPR technology to address cancers of various types. The CRISPR approach is another tool in the toolbox of biologists which can become a means for medical application.

As Cain and Boinett state:

The CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR associated proteins) adaptive immune system is widespread in bacteria and archaea and provides heritable protection against disruptive mobile genetic elements (MGEs), such as bacteriophages and plasmids. CRISPR loci contain a series of repetitive DNA motifs separated by spacer sequences; these spacers are derived from MGEs and incorporated after exposure to each new foreign element. The CRISPR transcript is processed into small CRISPR RNAs, which are displayed on Cas protein complexes, enabling RNA-guided degradation of the foreign DNA by Cas nucleases…. The flexibility and specificity of genome editing using CRISPR loci enables the efficient generation of mutated genotypes in diverse species. Furthermore, as CRISPR loci show strain-specific conservation at the nucleotide level, they are proving to be valuable markers for typing studies and, in conjunction with whole-genome sequencing, can provide insights into the phylogenetic relationships between different bacteria.

As reported in The Independent[19]:

The Crispr process was first identified as a natural immune defence used by bacteria against invading viruses. Last year, however, scientists led by Jennifer Doudna at the University of California, Berkeley, published a seminal study showing that Crispr can be used to target any region of a genome with extreme precision with the aid of a DNA-cutting enzyme called CAS9.

Since then, several teams of scientists showed that the Crispr-CAS9 system used by Professor Doudna could be adapted to work on a range of life forms, from plants and nematode worms to fruit flies and laboratory mice.

Earlier this year, several teams of scientists demonstrated that it can also be used accurately to engineer the DNA of mouse embryos and even human stem cells grown in culture. Geneticists were astounded by how easy, accurate and effective it is at altering the genetic code of any life form – and they immediately realized the therapeutic potential for medicine.

"The efficiency and ease of use is completely unprecedented. I'm jumping out of my skin with excitement," said George Church, a geneticist at Harvard University who led one of the teams that used Crispr to edit the human genome for the first time.

"The new technology should permit alterations of serious genetic disorders. This could be done, in principle, at any stage of development from sperm and egg cells and IVF embryos up to the irreversible stages of the disease," Professor Church said.

David Adams, a DNA scientist at the Wellcome Trust Sanger Institute in Cambridge, said that the technique has the potential to transform the way scientists are able to manipulate the genes of all living organisms, especially patients with inherited diseases, cancer or lifelong HIV infection.

[19] http://www.independent.co.uk/news/science/exclusive-jawdropping-breakthrough-hailed-as-landmark-in-fight-against-hereditary-diseases-as-crispr-technique-heralds-genetic-revolution-8925295.html

"This is the first time we've been able to edit the genome efficiently and precisely and at a scale that means individual patient mutations can be corrected," Dr Adams said.

"There have been other technologies for editing the genome but they all leave a 'scar' behind or foreign DNA in the genome. This leaves no scars behind and you can change the individual nucleotides of DNA – the 'letters' of the genetic textbook – without any other unwanted changes," he said.

The essence of the above is twofold. First it is the use of CRISPR as a mechanism in prokaryotes and possibly in eukaryotes. The second is an important observation that we now have another tool for the genetic engineering tool box. The observation that in genetic engineering that many of the "tools" are artifacts of nature should not be overlooked.

6.1.1 What is a CRISPR?

We will now examine in more detail what a CRISPR is and how it functions. Let us begin by examining it in a bit more detail. As Randow et al state:

In archaea and bacteria, for example, even adaptive forms of resistance—long considered the hallmark of vertebrates—contribute to cell autonomous immunity, as exemplified by the clustered regularly interspaced short palindromic repeats (CRISPR) system, which recognizes foreign DNA in a sequence-specific manner. In metazoans, cellular self-defense synergizes with the whole-body protection provided by traditional immunity to confer pathogen resistance. Here, professional immune cells patrol their environment in search of pathogens, whereas cell-autonomous immunity guards both individual immune and non-immune cells against the immediate threat of infection.

Cellular self-defense thus has the potential to confer antimicrobial protection on most, if not all, cells.…In bacteria, foreign DNA is sensed and destroyed by the CRISPR system and restriction endonucleases. Because recognition motifs for most restriction endonucleases occur frequently in the host's own genome, these enzymes are paired with matching methyltransferases, which modify host DNA to demarcate it as "self." In eukaryotic cells, rather than being modified, DNA is largely sequestered inside the nucleus, which fosters the detection of foreign DNA in other compartments and allows the deployment of enzymes that mutate and/or degrade DNA without risk to the host genome.

Thus as noted above, the original understanding was as a bacterial self-defense system. Now as Horvath and Barrangou state also concerning the original understanding:

Microbes have devised various strategies that allow them to survive exposure to foreign genetic elements. Although out-populated and preyed upon by abundant and ubiquitous viruses, microbes routinely survive, persist, and occasionally thrive in hostile and competitive environments. The constant exposure to exogenous DNA via transduction, conjugation, and transformation have forced microbes to establish an array of defense mechanisms that allow the cell to recognize and distinguish incoming "foreign" DNA, from "self " DNA and to survive exposure to invasive elements. These systems maintain genetic integrity, yet occasionally allow

exogenous DNA uptake and conservation of genetic material advantageous for adaptation to the environment.

Certain strategies, such as prevention of adsorption, blocking of injection, and abortive infection, are effective against viruses; other defense systems specifically target invading nucleic acid, such as the restriction-modification system (R-M) and the use of sugar-nonspecific nucleases. Recently, an adaptive microbial immune system, clustered regularly interspaced short palindromic repeats (CRISPR) has been identified that provides acquired immunity against viruses and plasmids.

They also state:

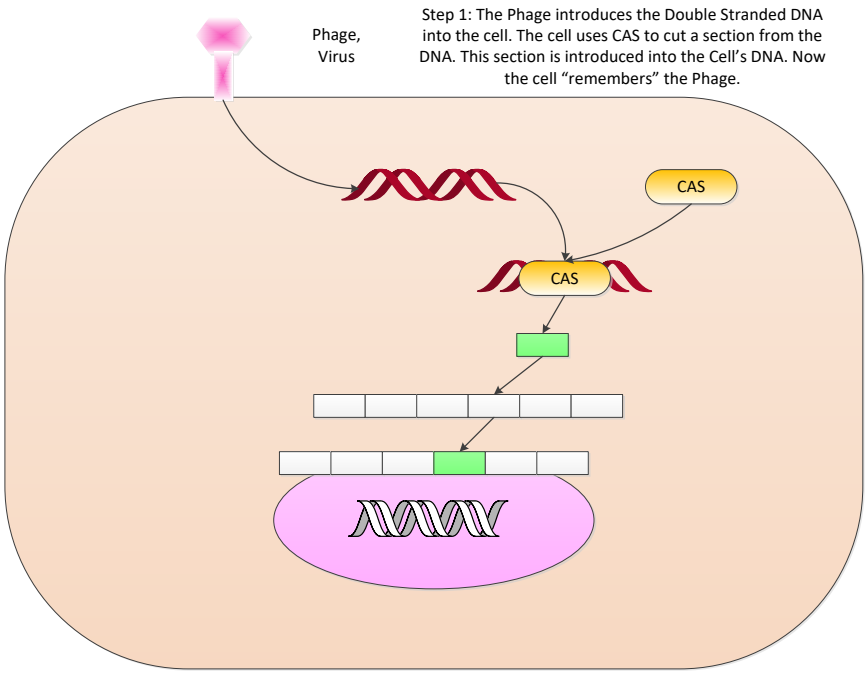
Microbes rely on diverse defense mechanisms that allow them to withstand viral predation and exposure to invading nucleic acid. In many Bacteria and most Archaea, clustered regularly interspaced short palindromic repeats (CRISPR) form peculiar genetic loci, which provide acquired immunity against viruses and plasmids by targeting nucleic acid in a sequence-specific manner.

These hypervariable loci take up genetic material from invasive elements and build up inheritable DNA-encoded immunity over time. Conversely, viruses have devised mutational escape strategies that allow them to circumvent the CRISPR/Cas system, albeit at a cost. CRISPR features may be exploited for typing purposes, epidemiological studies, host-virus ecological surveys, building specific immunity against undesirable genetic elements, and enhancing viral resistance in domesticated microbes.

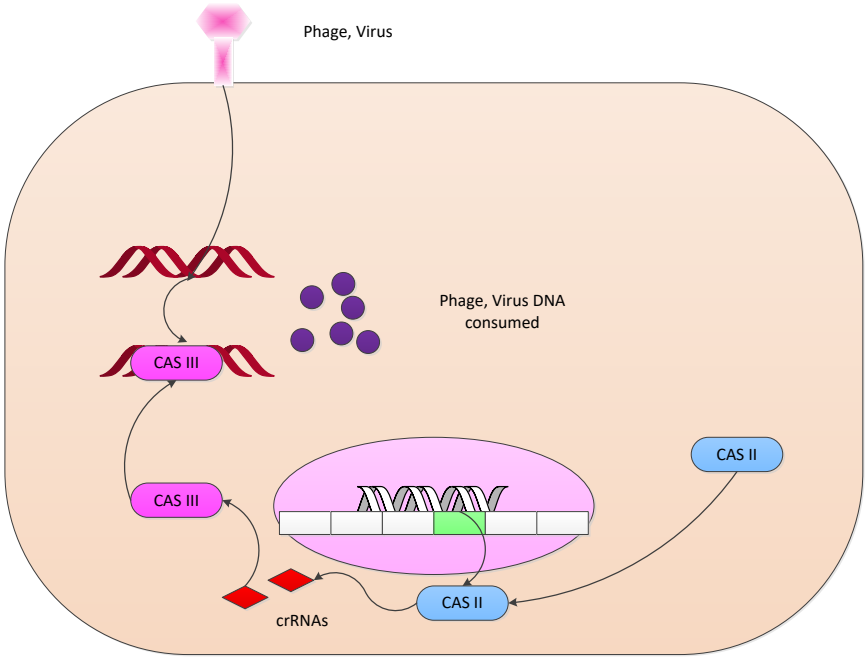
Thus we first examine how CRISPR-Cas functions in its primal environment and then we take this to human environments where we can use it as an added tool in our genetic engineering toolkit.

6.1.1.1 CRISPR Dynamics

We now examine some of the dynamics of the CRISPR system. We start with the use of CRISPR in a bacterial cell. We assume the cell is attacked by some viral phage and the phage sends its RNA/DNA into the cell in anticipation of replication within the host. Now from Horvath and Barrangou (as modified) we have the following description for this initial portion of the process as shown below:



The Cas protein recognizes the invading DNA and transports a portion of it to the nuclear DNA and inserts it into the cell's DNA. How specifically Cas does this task is not yet well understood. The when another phage with the same or frankly similar DNA invades again, then Cas II is activated and the section of the DNA activates a Cas II which then consumes the invading DNA.



Now the above process is a natural part of the day to day activities of bacteria. But it also is a paradigm for deal with eukaryotic cells, namely cutting and pasting genes into cells.

6.1.1.2 Types of CRISPR

From Jinek et al, they discuss the three types of CRISPR systems:

There are three types of CRISPR/Cas systems.

The type I and III systems share some overarching features: specialized Cas endonucleases process the pre-crRNAs, and once mature, each crRNA assembles into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA.

In contrast, type II systems process pre-crRNAs by a different mechanism in which a trans-activating crRNA (tracrRNA) complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded (ds) RNA specific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein. Cas9 is thought to be the sole protein responsible for crRNA-guided silencing of foreign DNA.

6.1.2 CRISPR Details

Current day biotechnology is in many ways a set of tools in a large tool box that handle the what and how of manipulating genes and their products. The tool and tool box metaphor is quite powerful and descriptive. The problem oftentimes is the why and also the integration of all of these elements from a technique to a technology.

In this brief paper we examine the CRISPR element less from that of a bench technique than as a technology that can be used in gene engineering. There is a mindset being explored that differs from that of the bench biologist. As an engineering approach one asks how can this technique be moved to a useful technology, and how deeply does one have to understand the underpinnings to use it effectively and safely.

One of the challenges of genetic engineering is the ability to select a specific gene and alter it, or add another gene or delete a gene. A key step in all of these is the ability to cut and paste at specific sites, at very specific sites. Now that one can read a gene in detail and when one knows what the desired result should be, then the cut and paste side is critical. Pasting is somewhat well known, especially if we have cut at the right location. CRISPR is a tool that does just that, it is a very accurate, fast, and low cost gene cutting tool.

In this note we examine its structure from a systematic perspective. This will help understand what factors are the key factors and what elements should be understood. This is not a note for a bench biologist, it is not meant to be comprehensive. Yet unlike many of the simplified descriptions in the media I try to provide adequate depth with breath of applications.

We also try to establish the "gene engineering" tools that this mechanism can support. Finally we discuss some of the concerns which have arisen in the use of CRISPRs.

To summarize, I refer to Mali et al who state:

Functioning of the type II CRISPR-Cas systems in bacteria.

Phase 1: in the immunization phase, the CRISPR system stores the molecular signature of a previous infection by integrating fragments of invading phage or plasmid DNA into the CRISPR locus as 'spacers'.

Phase 2: in the immunity phase, the bacterium uses this stored information to defend against invading pathogens by transcribing the locus and processing the resulting transcript to produce CRISPR RNAs (crRNAs) that guide effector nucleases to locate and cleave nucleic acids complementary to the spacer.

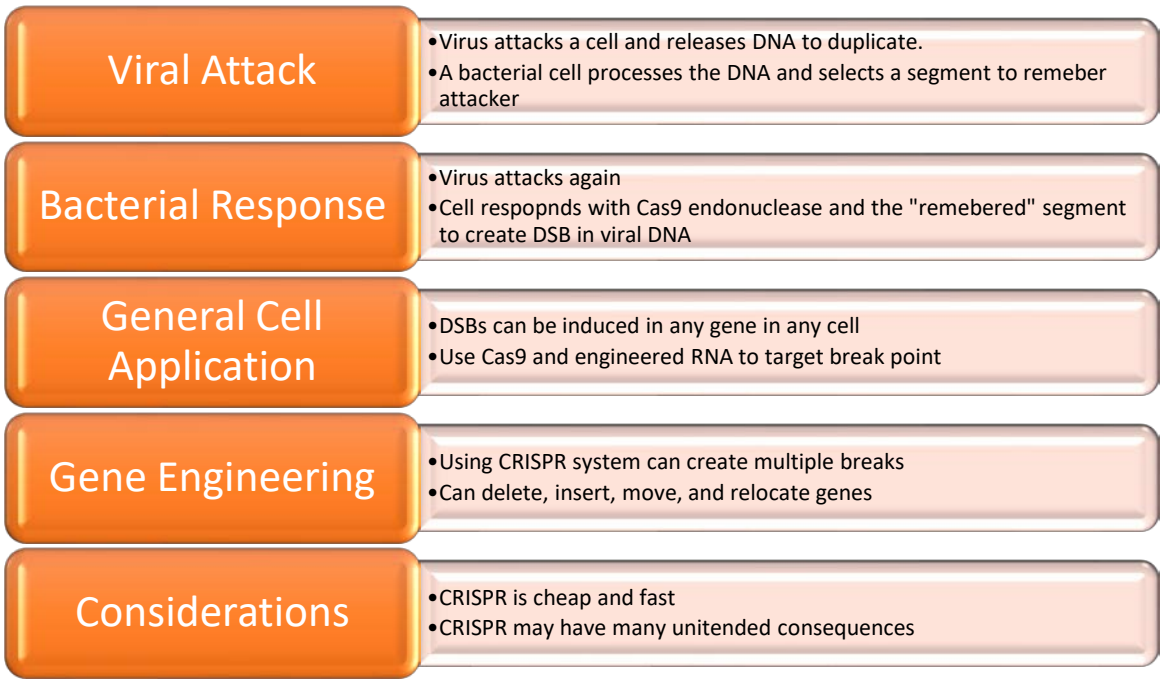
First, tracrRNAs hybridize to repeat regions of the pre-crRNA.

Second, endogenous RNase III cleaves the hybridized crRNAtracrRNA, and a second event removes the 5' end of the spacer, yielding mature crRNAs that remain associated with the tracrRNA and Cas9.

The complex cleaves complementary 'protospacer' sequences only if a PAM sequence is present.

Namely, this tool was seen developed in bacteria. The bacterium notes a section of the invading viral DNA, and then records that segment in its own DNA. Then when the virus attacks a second time, using the Cas9 nuclease protein produced by the bacteria then uses the RNA generated by the "remembered" sequences to attack the virus, and cut it so that it is made inoperable and it is digested.

Thus in the report we follow the following considerations:



6.1.2.1 Bacterial Immunology

The CRISPR phenomenon comes from examining bacteria and their quasi immune response to viral attacks. Simply stated;

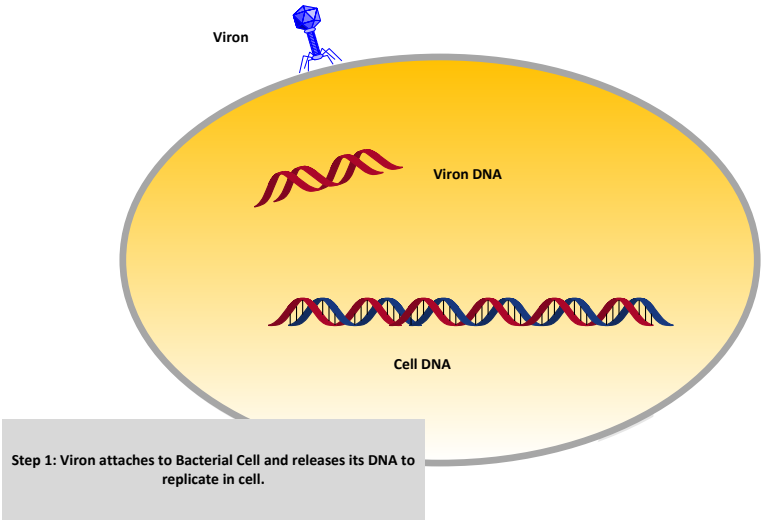
Bacteria have developed a technique where they can recognize a foreign viral DNA segment and then "attack" is with an enzyme and a targeted RNA segment that results in the foreign DNA being broken and becoming ineffective. This bacterial process effectively kills the DNA of the invader, stops its reproduction and induces an autophagy.

Now in discovering this process one then can take this same enzyme and modify the RNA that comes with it to match a location on some DNA we may be considering to manipulate and using this combo we can then cut DNA at a precise point anywhere we so desire. It is a powerful tool

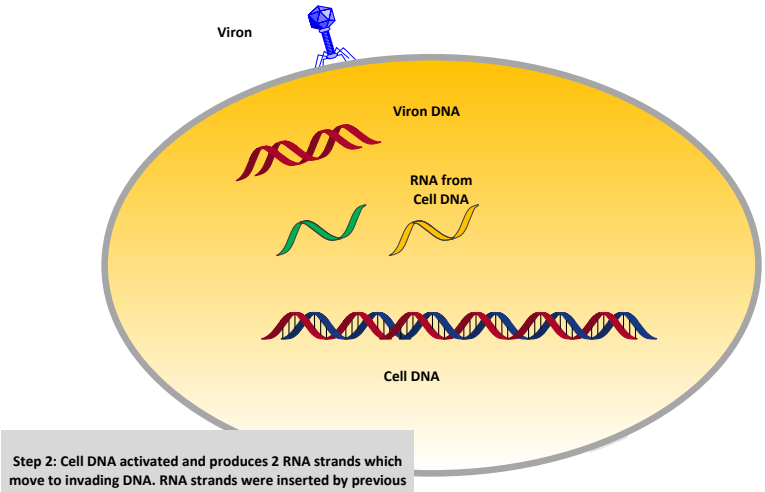
to cut DNA at a unique location. From there we can then add or delete DNA segments in a gene, in a somatic cell or in a germ line cell. It is fast and inexpensive and can be done in almost any lab.

Let us now begin with a viron attaching itself to a bacterium. We will assume that at some prior time some process has occurred where the bacteria had seen this for the first time. At this time that process is still a work in progress. But let us assume that this is a subsequent encounter and that in the process the bacteria has managed to record this prior encounter with a strand of DNA from that viron so that it can produce an RNA which is a map of some small segment of the viron's DNA.

This is a lot of assumptions but it is generally where we start with the tool. We just want to know what the tool does not how it was made or even how in any detail it does what it does. In many ways we are looking at tools as a technician, namely use this tool this way and get this result. Leave the details for someone else.

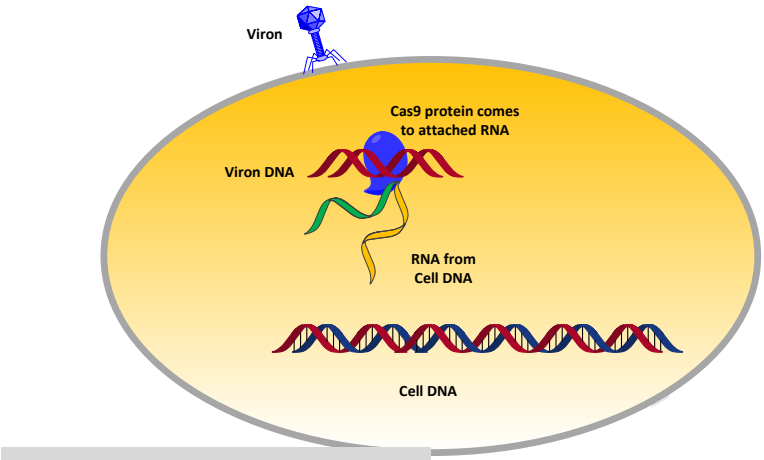


Now when the viron gets into the cell there is produced RNA from the bacteria that was RNA based upon a prior encounter with this viron. Namely this RNA released matches a segment of DNA in the viron. Also remember that a virus just wants to use a cell, any cell, to reproduce itself, which frankly is just reproducing its DNA (or RNA). If the bacteria can use this knowledge of the attacker then what can it do to stop the reproduction, and potentially the organism's death.



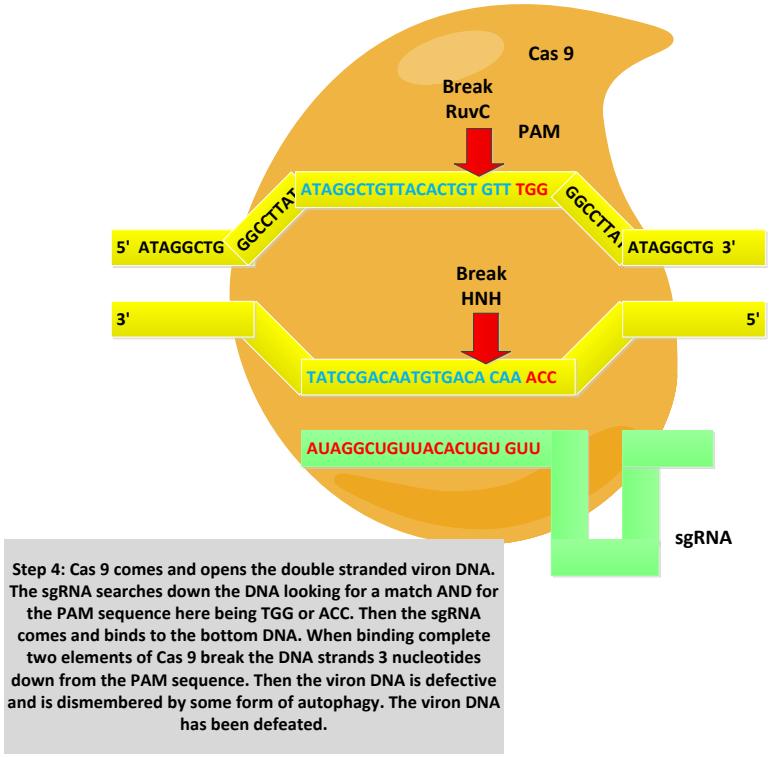
Step 2: Cell DNA activated and produces 2 RNA strands which move to invading DNA. RNA strands were inserted by previous attack by a viron with such DNA.

Now the RNA segments migrate to the viron DNA and along with a protein called cas9. The cas9 protein is the secret sauce of this tool.

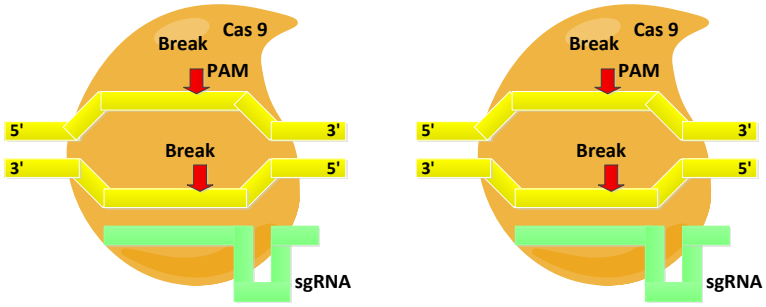


Step 3: Cas9 protein is released and combines with viron DNA, combined RNA to create a CRISPR Cas9 cut of the viron DNA to be shown next in detail.

The details of the operation are depicted in the graphic below. One must recall that this tool works but its operation is not fully understood. The Cas9 protein surrounds the desired site which has been selected by a combination of two factors. The first is the PAM sequence, in this case 3 nucleotides, nt, which act as a marker and then a 20 nt long matching strand down from the PAM. This key determines where the break occurs. In a bacteria's immune like response it needs both, the PAM to be certain it does not kill itself and the 20 nt strand which gives a good marker for a specific site. In effect we have 23 nt for specific targeting. In genetic engineering cases we select the PAM as specified by the Cas9 source and then engineer the sgRNA element. That yields a specific break site at 3 nt down from the end of the PAM. The two Cas9 fragments, RuvC and HNH are what cause the break.



The example below extends the above example to a double stranded break.



6.1.2.2 crRNA and tracrRNA

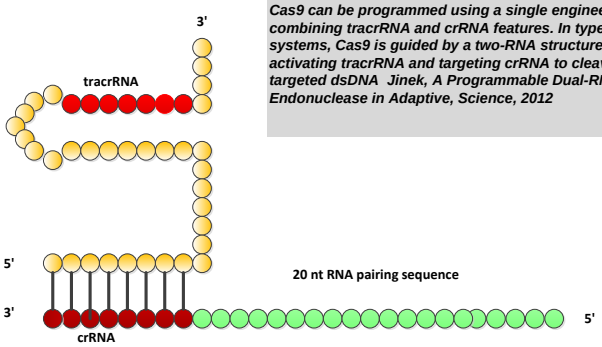
The two RNA segments, crRNA and tracrRNA can be configured in several ways. But they are the targets elements that are used to select where the break is to be. And once selected it is usually a double strand break. However single strand breaks can be accomplished as well.

As Jinek et al state:

In the expression and interference phases, transcription of the repeat-spacer element into precursor CRISPR RNA (pre-crRNA) molecules followed by enzymatic cleavage yields the short ***crRNAs that can pair with complementary protospacer sequences of invading viral or plasmid targets****. Target recognition by crRNAs directs the silencing of the foreign sequences by means of Cas proteins that function in complex with the crRNAs ….*

There are three types of CRISPR/Cas systems. The type I and III systems share some overarching features: specialized Cas endonucleases process the pre-crRNAs, and once mature, each crRNA assembles into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA. In contrast, type II systems process precrRNAs by a different mechanism in which a ***trans-activating crRNA (tracrRNA)*** *complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded (ds) RNA specific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein. Cas9 is thought to be the sole protein responsible for crRNA-guided silencing of foreign DNA*

We demonstrate one variation of this below. Note the tracrRNA and its binding with crRNA and the 20 nucleotide ("nt") sequence which will select out the point at which we desire a break to be made.



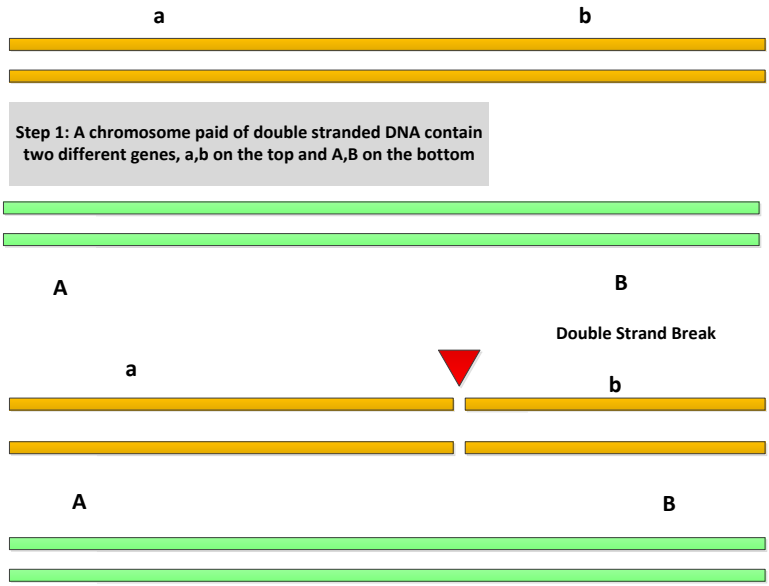
Cas9 can be programmed using a single engineered RNA molecule combining tracrRNA and crRNA features. In type II CRISPR/Cas systems, Cas9 is guided by a two-RNA structure formed by activating tracrRNA and targeting crRNA to cleave site-specifically – targeted dsDNA Jinek, A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive, Science, 2012

6.1.2.3 Gene Engineering

No we ask; given a break at the right point what do we do next? That is the beginning of gene engineering. We briefly examine homologous repair, a somewhat well understood process, which uses the other chromosome as a template. The use of templates may also be done to insert new genes as well.

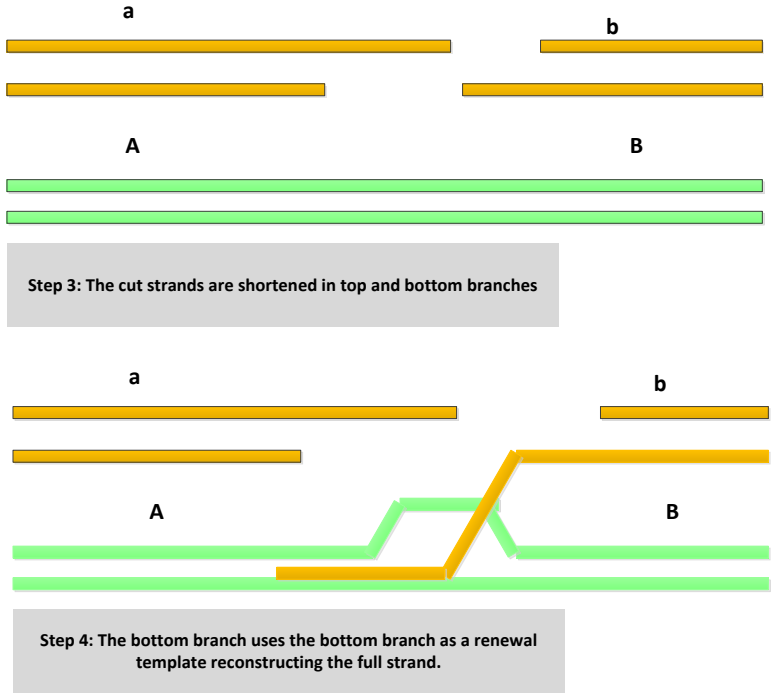
1. Let us start with a chromosome pair, one from each parent. We show this below.

2. Now we assume a double strand break, DSB, occurs on the top chromosome pair. We show this below:



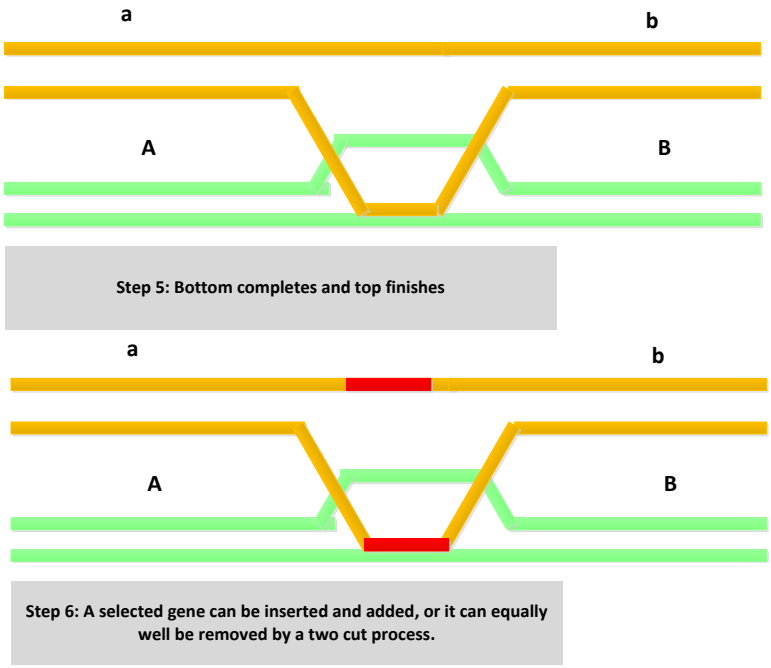
3. Next we see a shortening of strands as shown below;

4. Then we see and elongation and use of the strand in the uncut DNA as a template. This can be used for other templates as insertion mechanisms.



5. We see both top and bottom expanding and a crossover occurring.

6. We can see this also as an insertion mechanism.



Now this is a simple reconstruction of the process. Details are in Watson et al.

6.1.3 Applications

There are a large set of applications for this tool. We consider some here but it is anticipated that there will be many more. There is a balance between correcting gene defects in such disorders as muscular dystrophy, thalassemia, sickle cell anemia, or cystic fibrosis, and cancers such as those involving BRCA genes. There are also applications in the field of plant genetics which are extensive. Plants are pluripotent; that is, a single cell can regenerate almost any plant. Thus adding or extracting a gene can dramatically change a plant's characteristics. We have examined some of these opportunities elsewhere for horticultural plants.

6.1.3.1 Gene Extraction

The simplest application is gene extraction. Using two DSBs at the desired locations we can accurately extract a gene.

6.1.3.2 Gene Insertion

Gene insertion is a major step. A template must be available and an insertion point specified. It is also important to understand the location of any promoter genes or suppressor genes. Just inserting may not always work. There is also the issue of methylation and acetylation as well as miRNA interference.

6.1.3.3 Somatic Applications

As the body matures or as a result of a genetic defect, we often see genetic changes which result in less than benign results.

6.1.3.4 Germline Applications

This is the most concerning application. Recently a group of researchers have indicated their concern and we shall discuss it later. However, one can take a sperm and ovum, cut-and-paste a new set of genes, and then allow them to combine and we have developed putatively a new species.

6.1.4 Types of Applications

There have been a multiplicity of papers on various applications We have indicated some general ones above but the Zhang Lab at MIT has performed a great deal worth examining

From the work of Cox et al,

The specific type of genome editing therapy depends on the nature of the mutation causing disease.

(a) In gene disruption, the pathogenic function of a protein is silenced by targeting the locus with NHEJ. Formation of indels in the gene of interest often results in frameshift mutations that create premature stop codons resulting in a nonfunctional protein product or nonsense-mediate decay of transcripts, suppressing gene function. Gene disruption may also be used to introduce protective loss of- function mutations into wild-type genes to generate a therapeutic effect (Box 1).

(b) In NHEJ gene correction, two DSBs targeted to both sides of a pathogenic expansion or insertion may be resolved by NHEJ, causing a therapeutic deletion of the intervening sequences. This form of treatment would require multiplexed targeting of disease-causing mutations.

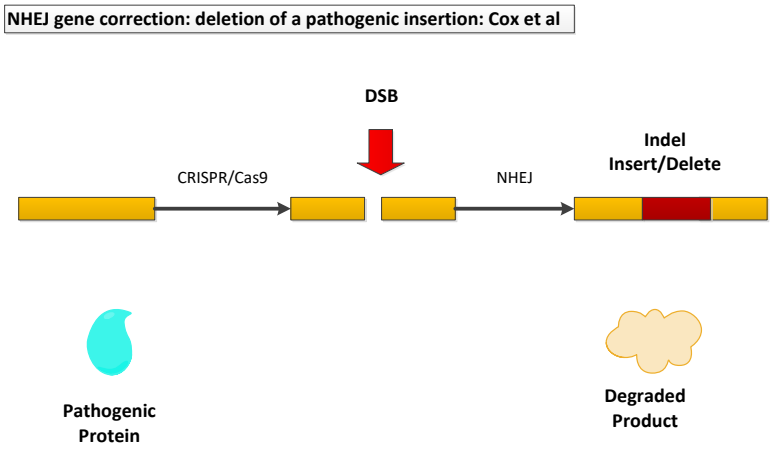
(c) HDR gene correction can be used to correct a deleterious mutation. A DSB is induced near the mutation site in the presence of an exogenously provided, corrective HDR template. HDR repair of the break site with the exogenous template corrects the mutation, restoring gene function.

(d) An alternative to gene correction is HDR gene addition, which introduces a therapeutic transgene into a predetermined locus. This may be the native locus, a safe harbor locus or a non-native locus. A DSB is induced at the desired locus, and an HDR template containing sequence similarity to the break site, a promoter, a transgene and a polyadenylation sequence is introduced to the nucleus. HDR repair restores gene function in the target locus, albeit without true physiological control over gene expression.

We graphically demonstrate some of these below:

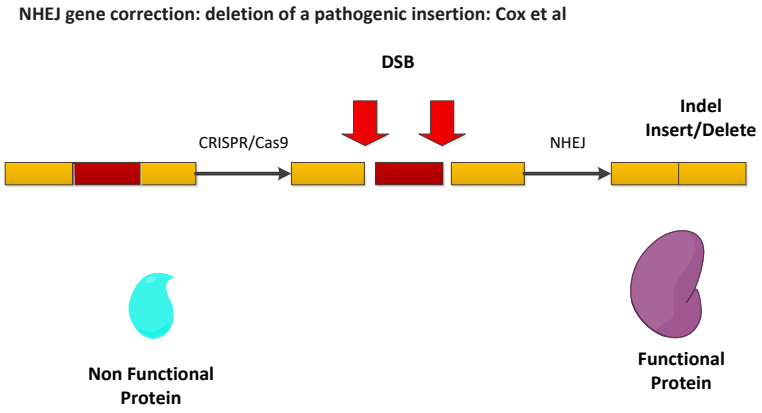
6.1.4.1 Deletion

The first cases below are a non-homologous break and joining of some insertion/deletion ("indel") to degrade the production of a protein. This is a simple double stranded break.



6.1.4.2 Correction

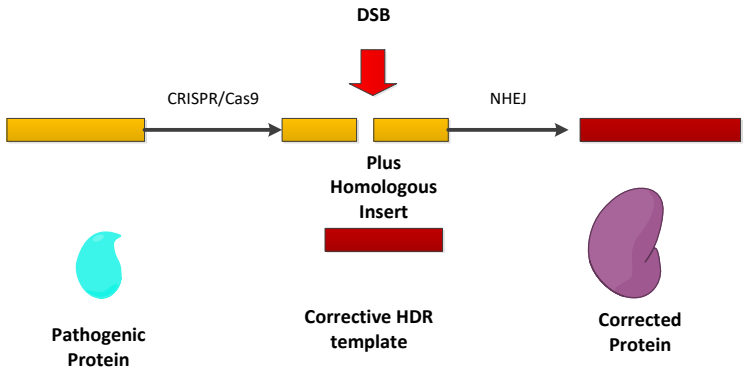
The second is again one using a DSB but this time a specific section is deleted and it requires two DSBs to be initiated. This may be an application where we seek a functional protein. It may be possible to employ this in a fusion gene process as well.



6.1.4.3 Homologous Correction

The next two applications are for homologous changes where we have a template to reconstruct the gene of the type we have previously discussed. The example below is a simple correction process with a single DSB.

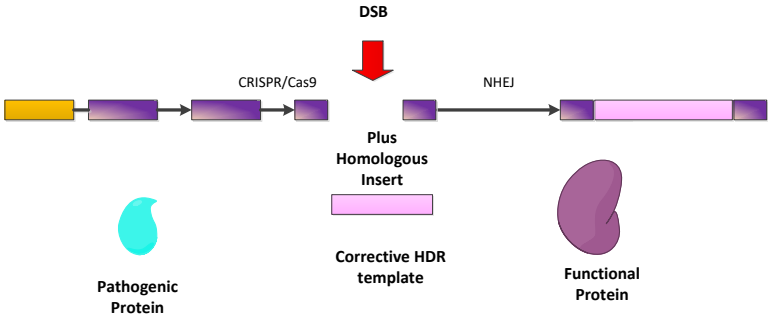
HDR gene correction: correct a deleterious mutation: See Cox et al



6.1.4.4 Homologous Insertion

The final example below is for a complicate deletion and insertion. Again it uses a template and a homologous rebinding.

HDR gene addition: introduce a therapeutic gene: See Cox et al



6.1.4.5 Ex Vivo and In Vivo

There are several dimensions in applying this tool to humans. They can be inserted ex vivo or in vivo. As Cox et al state:

… in ex vivo editing therapy, cells are removed from a patient being treated, edited and then re-engrafted. For this mode of therapy to be successful, the target cells must be capable of surviving outside the body and homing back to target tissues after transplantation.

… in vivo therapy involves genome editing of cells in situ. For in vivo systemic therapy, delivery agents that are relatively agnostic to cell identity or state would be used to effect editing in a wide range of tissue types. Alternatively, targeted in vivo therapy may also be achieved through targeted local injection of viral vectors to the affected tissue or through the systemic injection of viral vectors with inherent tropism for specific diseased tissues, such as the eye brain, or muscle.

Now we can see this as applied in somatic or germ line cells.

	Ex Vivo	***In Vivo***
Somatic	One may consider this approach in certain hematopoietic cell lines.	Insertion into targeted somatic cells of modified genes may produce or delete expressions of genes. Various means of insertion are possible.
Germ Line	This is a somewhat routine procedure for certain reproductive processes. However the viability and sustainability of such cells may have issues.	Each of the germ line cells may be separately dealt with insertions or deletions and then the two combined. This method also permits the insertion of CRISPR Cas9 genes themselves to assure continual propagation of the desired change. However this may be a very complex approach since it deals with in vivo germ line cells where injecting the cells may not function completely.

6.1.5 CRISPR and Cancer Treatment

Thus one may ask if one knows that some gene has been the cause of a cancer, can we then treat the cells with a CRISPR-Cas system to delete the gene and replace it with a normal wild type. If we have a procedure to do this then perhaps this is a therapeutic approach. It does, of course beg the question of how this is accomplished even if we have the chimeric Cas delivery system. We also must ask if we have identified all the genes. There are also many other such questions. Yet this has become a focal point of interest.

In a recent paper by Yin et al the authors discuss the delivery of a CRISPR-Cas9 mediated cutting and reintroduction of a gene into liver cells by means of an injection process. The result was conversion of the errant gene cells into normal wild type cells. They utilized the backwards flow of the CRISPER-Cas9 approach for cutting and injection. This potentially paves the way for substantial progress in alternative targeted gene replacement and return to normal states. As Yin et al state:

We demonstrate CRISPR-Cas9–mediated correction of a Fah mutation in hepatocytes in a mouse model of the human disease hereditary tyrosinemia. Delivery of components of the CRISPR-Cas9 system by hydrodynamic injection resulted in initial expression of the wild-type Fah protein in ~1/250 liver cells. Expansion of Fah-positive hepatocytes rescued the body weight loss phenotype. Our study indicates that CRISPR-Cas9–mediated genome editing is possible in adult animals and has potential for correction of human genetic diseases.

From Gene News[20] we have a more detailed discussion worthy of note regarding the above recent report:

MIT scientists report the use of a CRISPR methodology to cure mice of a rare liver disorder caused by a single genetic mutation. They say their study … offers the first evidence that this gene-editing technique can reverse disease symptoms in living animals. CRISPR, which provides a way to snip out mutated DNA and replace it with the correct sequence, holds potential for treating many genetic disorders, according to the research team.

"What's exciting about this approach is that we can actually correct a defective gene in a living adult animal," says Daniel Anderson, Ph.D., the Samuel A. Goldblith associate professor of chemical engineering at MIT, a member of the Koch Institute for Integrative Cancer Research, and the senior author of the paper.

The recently developed CRISPR system relies on cellular machinery that bacteria use to defend themselves from viral infection. Researchers have copied this cellular system to create gene-editing complexes that include a DNA-cutting enzyme called Cas9 bound to a short RNA guide strand that is programmed to bind to a specific genome sequence, telling Cas9 where to make its cut.

At the same time, the researchers also deliver a DNA template strand. When the cell repairs the damage produced by Cas9, it copies from the template, introducing new genetic material into the genome. Scientists envision that this kind of genome editing could one day help treat diseases such as hemophilia, Huntington's disease, and others that are caused by single mutations.

For this study, the researchers designed three guide RNA strands that target different DNA sequences near the mutation that causes type I tyrosinemia, in a gene that codes for an enzyme called FAH. Patients with this disease, which affects about 1 in 100,000 people, cannot break down the amino acid tyrosine, which accumulates and can lead to liver failure. Current treatments include a low-protein diet and a drug called NTCB, which disrupts tyrosine production.

In experiments with adult mice carrying the mutated form of the FAH enzyme, the researchers delivered RNA guide strands along with the gene for Cas9 and a 199-nucleotide DNA template that includes the correct sequence of the mutated FAH gene.

"Delivery of components of the CRISPR-Cas9 system by hydrodynamic injection resulted in initial expression of the wild-type Fah protein in ~1/250 liver cells," wrote the investigators. "Expansion of Fah-positive hepatocytes rescued the body weight loss phenotype."

While the team used a high pressure injection to deliver the CRISPR components, Dr. Anderson envisions that better delivery approaches are possible. His lab is now working on methods that may be safer and more efficient, including targeted nanoparticles.

[20] http://www.genengnews.com/gen-news-highlights/crispr-reverses-disease-symptoms-in-living-animals-for-first-time/81249682/

The above described an interesting I vivo approach to the editing and insertion of a specific gene in a specific location. Although this is of interest, it is limited to a very specific site and also using a difficult delivery mechanism.

As to more extensive editing capabilities we examine Zhang et al who state:

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system provides a robust and multiplexable genome editing tool, enabling researchers to precisely manipulate specific genomic elements, and facilitating the elucidation of target gene function in biology and diseases. CRISPR/Cas9 comprises of a non-specific Cas9 nuclease and a set of programmable sequence-specific CRISPR RNA (crRNA), which can guide Cas9 to cleave DNA and generate double-strand breaks at target sites. Subsequent cellular DNA repair process leads to desired insertions, deletions or substitutions at target sites.

The specificity of CRISPR/Cas9-mediated DNA cleavage requires target sequences matching crRNA and a protospacer adjacent motif (PAM) locating at downstream of target sequences. Here, we review the molecular mechanism, applications and challenges of CRISPR/Cas9-mediated genome editing and clinical therapeutic potential of CRISPR/Cas9 in future.

The above stresses the strong point of CRISPR-Cas9, namely its specificity. It can target specific DNA, assuming we know what to target. It can then replace that with a substitute, assuming we know that the substitute does no harm and in fact is positively therapeutic. From Pandika we have[21] the following lengthy discussion regarding the evolution of this specific result:

Because a Nobel Prize winner says this breakthough is better than his breakthrough.
Jennifer Doudna has always had an explorer's spirit. It's what led the UC Berkeley molecular and cell biology professor to engineer a cheaper, easier way to correct DNA defects. Her game-changing technology takes a mysterious bacterial genetic code and transforms it into a powerful tool for cutting and pasting bits of genetic material – meaning not only could the entire field of gene therapy be revived, but her genome-editing tool could one day be used to treat a range of diseases, from cancer and AIDS to hereditary disorders like Down syndrome and Huntington disease.

Every time we see some new tool for the toolkit the immediate tendency is to label it as a cure for cancer.

Most scientists weren't even aware of these so-called CRISPRs, much less their function. But Doudna suspected they hid a crucial purpose.…The bacterial enzyme Cas9 is the engine of RNA-programmed genome engineering in human cells. Doudna unearthed the first clue when she found that a protein called Cas9 acts like a pair of molecular scissors…

"I wasn't actively trying to go in any particular direction," she said. That willingness to wander, to maybe even get a little lost, could be how she was able to make a creative break from earlier

[21] http://www.ozy.com/rising-stars-and-provocateurs/jennifer-doudna/4690.article

genome-editing technologies. Doudna "certainly didn't set out to discover a genome editing tool by any stretch of the imagination." It all began with a puzzle she couldn't resist solving, thanks largely to her father. When Doudna was growing up, the literature professor got her hooked on one of his favorite pastimes —decoding short pieces of encrypted text, or cryptograms.

In 2005, a colleague presented Doudna with a genetic cryptogram — weird repetitive RNA sequences tucked in the genomes of many of the bacteria she studied. Most scientists weren't even aware of these so-called CRISPRs, much less their function. But Doudna suspected they hid a crucial purpose.

Sure enough, scientists discovered that CRISPRs played an important role in immunity: they recognize the DNA of viral invaders for the bacteria to chop up and fight off. But how did this search-and-destroy mechanism work? Teaming up with Umea University molecular biologist Emmanuelle Charpentier, Doudna unearthed the first clue when she found that a protein called Cas9 acts like a pair of molecular scissors. A CRISPR RNA fragment hooks up with Cas9 to precisely target the DNA of an invading virus, which it then cuts and destroys.

Here's where it gets really complicated. Martin Jinek, a postdoctoral researcher in Doudna's lab, found that Cas9 in bacteria needs two RNA guide strands – this sent the gears in their heads turning. What if they could engineer the system to require only a single, programmable RNA strand? Then biologists could use it to easily target and cut any DNA sequence. Doudna felt "a chill of excitement." Maybe they could link the two RNA strands into one, and loop it in on itself—mimicking a double-stranded structure. Those chills were warranted: Doudna's lab and other groups successfully used this simplified CRISPR system to modify genes in bacteria, plant and animal cells.

One early form of CRISPR-based gene therapy could involve editing the genes responsible for blood disorders like sickle-cell anemia in bone marrow cells, growing them into mature blood cells and injecting them back into patients.

However, the application needs a more effective insertion system. It also needs to demonstrate that it does not wander and affect other genes.

Little more than a year after Doudna first described CRISPR in the journal Science, the cut-and-paste technology has yielded promising results in labs around the world. Last month, researchers from the Netherland's Utrecht institute reported in Cell Stem Cell that CRISPR corrected the gene mutation responsible for cystic fibrosis in stem cells developed from two children with the life-threatening disease. Doudna believes a clinical trial of CRISPR-based gene therapy could begin in less than a decade.

As is all too often the case, any prediction of clinical application may be much too speculative. Single gene targeting may become the first step, albeit even there one must be cautious.

Doudna experienced "many frustrations" getting CRISPR to work in human cells. But she knew if she succeeded, CRISPR would be "a profound discovery" — and maybe even a powerful gene therapy technique.

We knew if the system could be made to work in human cells, it would be a really profound discovery.

"I hope you're sitting down," an excited colleague told Doudna in an unexpected phone call. "CRISPR is turning out to be absolutely spectacular in [Harvard geneticist] George Church's hands." He had even gotten it to work in human cells. Thrilled, Doudna immediately contacted Church. They shared their results, and both published studies in January 2013 showing that CRISPR can cut, delete and replace genes in human cells. University of Massachusetts biologist Craig Mello, who shared the 2006 Nobel Prize for another genome editing tool, hails Doudna's CRISPR technique as a "tremendous breakthrough," even admitting that "in many ways it's better" than his own technique.

Other techniques can also edit genes at specific DNA regions. But they require scientists to engineer a separate protein for each target site. In contrast, CRISPR only needs the Cas9 protein, allowing it to correct multiple defects at once. Besides being cheaper and easier to use, CRISPR is also much more precise, reducing the risk of off-target modifications introducing dangerous mutations. As a result, it could help revive the gene therapy field, whose early clinical failures — including patient deaths — led some to dismiss it as overhyped.

That doesn't mean CRISPR is perfect, though. While it's extremely precise, it occasionally modifies DNA at similar sites elsewhere in the genome instead of the target gene. Understanding and exploiting how Cas9 avoids these close matches "is an active area of investigation," Doudna said. Still, CRISPR is "a real game-changer," Mello told the Independent. "It's incredibly powerful."[22]

Indeed the observations above detail some of the powers of CRISPR-Cas9 complex.

Now one would think that perhaps this could become a therapeutic as applied to various cancers. Consider it use as a kinase inhibitor in CML. Would it work there by targeting the aberrant kinases? What of an application in melanoma with a BRAF V400 mutation? Can we cut and paste back the proper genetic sequence? If so, how do we deliver the elements of the process, especially in a metastatic case? Furthermore, how do we determine what genes must be modified, and does that mean that we not only customize it for a patients but also for cells? Finally how do we know that there are not some deleterious sequellae from this cutting and pasting process, what if we "miss" the gene in some cell and start an secondary malignancy?

These are all reasonable questions that lead us to examine the CRISPR process in further detail

As recently stated by Stephen et al:

We are also optimistic that completely different approaches to treating cancer will contribute to eliminating Ras cancers, including new ways of knocking down/out genes using RNAi and

[22] http://www.independent.co.uk/news/science/exclusive-jawdropping-breakthrough-hailed-as-landmark-in-fight-against-hereditary-diseases-as-crispr-technique-heralds-genetic-revolution-8925295.html

CRISPR technologies and delivering these payloads to tumors, as well as new ways of deploying the immune system.

In this respect, it is noteworthy that anti-CTLA-4 therapy appears to be equally effective in treating melanoma driven by N-Ras or B-Raf; therefore, Ras cancers may not be excluded from these approaches as they have been from others. All of these considerations lead us to be optimistic about future prospects of finally delivering the knockout punch.

As Way et al state:

Synthetic biology is a young discipline with the declared goal of rationally engineering biological systems through approaches similar to those used by engineers to build bridges and send people to the moon. This field has rapidly developed over the past 15 years from its initial conceptualization by a few academics and government program managers into a sizeable field whose meetings attract large numbers of participants. Recently, new tools have emerged that should allow specific integration at desired sites in the genome. For example, methods based on zinc-finger, TALE, and CRISPR fusions to nucleases can be used to generate double-strand breaks at specific sites in the genome. The questions remain—where should we integrate, and how can we avoid effects of adjacent sequences?

6.1.6 Observations

Having given a high level description of this tool we can make several key observations. Amongst them is the recent concern as to the potential abuse of the process.

CRISPR Cas 9 is a new technique to cut and splice genes. We had written about it about a year ago regarding its use in cancer treatment and also regarding the patent so quickly issues. Now David Baltimore, a highly respected scientist, and colleagues have in Science suggested a prudent set of steps as to its use in humans. It is reminiscent of the concerns some 40 years ago regarding recombinant DNA.

6.1.6.1 Controls to Use

Recently several groups of researchers have become concerned regarding the unintended consequences of CRISPRs. For example David Baltimore et al recommend:

In the near term, we recommend that steps be taken to:

1) Strongly discourage, even in those countries with lax jurisdictions where it might be permitted, any attempts at germline genome modification for clinical application in humans, while societal, environmental, and ethical implications of such activity are discussed among scientific and governmental organizations. (In countries with a highly developed bioscience capacity, germline genome modification in humans is currently illegal or tightly regulated.) This will enable pathways to responsible uses of this technology, if any, to be identified.

2) Create forums in which experts from the scientific and bioethics communities can provide information and education about this new era of human biology, the issues accompanying the risks and rewards of using such powerful technology for a wide variety of applications including the potential to treat or cure human genetic disease, and the attendant ethical, social, and legal implications of genome modification.

3) Encourage and support transparent research to evaluate the efficacy and specificity of CRISPR-Cas9 genome engineering technology in human and nonhuman model systems relevant to its potential applications for germline gene therapy. Such research is essential to inform deliberations about what clinical applications, if any, might in the future be deemed permissible.

4) Convene a globally representative group of developers and users of genome engineering technology and experts in genetics, law, and bioethics, as well as members of the scientific community, the public, and relevant government agencies and interest groups—to further consider these important issues, and where appropriate, recommend policies.

Baltimore et al have a significant point. Not only can this be significant on a person by person basis but it also has the potential to be weaponized. The technology is out there, thousands are now proficient in it, the cost is low and the means for distribution is high.

Clearly a sensible effort in collaboration with others is essential. The problem is that with much of science, the genie is out of the box.

The system used by bacteria to defend against a virus attacking is the CRISPR Cas 9 system. An interesting use of a protein, enzyme, and a DNA segment that can open DNA at desired locations and cut and insert new segments of DNA. We have been discussing this for well over a year now and have discussed its potential and its risks.

Now along comes researchers who instead of doing this in somatic cells do it in germline cells, thus changing the potentially maturing entity. Thus each cell has this changed gene or genes.

In a recent Nature article the authors state[23]:

There are grave concerns regarding the ethical and safety implications of this research. There is also fear of the negative impact it could have on important work involving the use of genome-editing techniques in somatic (non-reproductive) cells....In our view, genome editing in human embryos using current technologies could have unpredictable effects on future generations. This makes it dangerous and ethically unacceptable. Such research could be exploited for non-therapeutic modifications. We are concerned that a public outcry about such an ethical breach could hinder a promising area of therapeutic development, namely making genetic changes that cannot be inherited. At this early stage, scientists should agree not to modify the DNA of human reproductive cells. Should a truly compelling case ever arise for the therapeutic benefit of germ-line modification, we encourage an open discussion around the appropriate course of action.

[23] http://www.nature.com/news/don-t-edit-the-human-germ-line-1.17111

Now this point is well made. Germline cell changes introduce all sorts of issues. Not only is there the issue of what this new gene will do, we hardly have begun to understand gene interactions, but the issues of epigenetic factors such as methylation dramatically change the risks.

Then again you do have the techno-advocates in Technology Review, who state[24]:

When I visited the lab last June ... proposed that I speak to a young postdoctoral scientist named ..., a Harvard recruit from Beijing who'd been a key player in developing a new, powerful technology for editing DNA, called CRISPR-Cas9. With ...had founded a small company to engineer the genomes of pigs and cattle, sliding in beneficial genes and editing away bad ones. As I listened to ..., I waited for a chance to ask my real questions: Can any of this be done to human beings? Can we improve the human gene pool? The position of much of mainstream science has been that such meddling would be unsafe, irresponsible, and even impossible. But ... didn't hesitate. Yes, of course, she said. In fact, the Harvard laboratory had a project to determine how it could be achieved.

She flipped open her laptop to a PowerPoint slide titled "Germline Editing Meeting." Here it was: a technical proposal to alter human heredity. "Germ line" is biologists' jargon for the egg and sperm, which combine to form an embryo. By editing the DNA of these cells or the embryo itself, it could be possible to correct disease genes and to pass those genetic fixes on to future generations. Such a technology could be used to rid families of scourges like cystic fibrosis. It might also be possible to install genes that offer lifelong protection against infection, Alzheimer's, and ... told me, maybe the effects of aging.

These would be history-making medical advances that could be as important to this century as vaccines were to the last.

The problem is, as the writers in Nature and in Science led by David Baltimore, has noted, the germ line modifications could be unwieldy.

Just because we have a new technology is no reason to let is loose. The problem with this technology is that it not only can be weaponized but that it can be done in a basement lab. This not building a nuclear weapon. This is potentially setting the world afire.

The again there is the issue of Government regulation. In an interesting piece in Xconomy the author remarks[25]:

But researchers' and investors' fear that a patchwork of regulation would cripple biotechnology in the United States did not disappear right away. Biologist Thomas Maniatis of Harvard left his

[24] http://www.technologyreview.com/featuredstory/535661/engineering-the-perfect-baby/
[25] http://www.xconomy.com/boston/2015/03/26/amid-gene-editing-worry-a-return-to-biotechs-1st-asilomar-moment/2/

home lab to work on the techniques in tighter-security conditions at Cold Spring Harbor Laboratory in New York.

Others went abroad. Biogen, founded in 1978, put its first major lab in Geneva, Switzerland. This was a time of intense concern about environmental dangers from the chemical industry in particular and science in general. It took some years for biologists to gain respect among local state, and federal officials for their sense of responsibility in the recombinant DNA maelstrom of the mid-1970s. But politicians did accept that biotechnology was a significant new industry that other countries, like Japan, might seize if America dropped the ball.

A valid point, but in the 70s we worried about errant scientists. Now we are terrified about terrorist post docs! One wonders what would be worse; the Government Regulators or the Terrorist?

6.1.7 Current Applications

There are many areas where this technology may have immediate use. There is a report in Genome Research of CRISPR being used to correct β-Thalassemia. They state[26]:

β-thalassemia, one of the most common genetic diseases worldwide, is caused by mutations in the human hemoglobin beta (HBB) gene. Creation of human induced pluripotent stem cells (iPSCs) from β-thalassemia patients could offer an approach to cure this disease. Correction of the disease-causing mutations in iPSCs could restore normal function and provide a rich source of cells for transplantation.

In this study, we used the latest gene-editing tool, CRISPR/Cas9 technology, combined with the piggyBac transposon to efficiently correct the HBB mutations in patient-derived iPSCs without leaving any residual footprint.

No off-target effects were detected in the corrected iPSCs, and the cells retain full pluripotency and exhibit normal karyotypes. When differentiated into erythroblasts using a monolayer culture, gene-corrected iPSCs restored expression of HBB compared to the parental iPSCs line.

Our study provides an effective approach to correct HBB mutations without leaving any genetic footprint in patient-derived iPSCs, thereby demonstrating a critical step toward the future application of stem cell-based gene therapy to monogenic diseases.

We have considered Cancer applications in a separate note in 2014. Cox et al present the following summary Table:

[26] http://genome.cshlp.org/content/early/2014/07/30/gr.173427.114.abstract

Disease type	*Nuclease platform*	*Therapeutic strategy*
Hemophilia B	ZFN	HDR-mediated insertion of correct gene sequence
HIV	ZFN and CRISPR	NHEJ-mediated inactivation of CCR5
Duchene muscular dystrophy (DMD)	CRISPR and TALEN	NHEJ-mediated removal of stop codon, and HDR-mediated gene correction
Hepatitis B virus (HBV)	TALEN and CRISPR	NHEJ-mediated depletion of viral DNA
SCID	ZFN	HDR-mediated insertion of correct gene sequence
Cataracts	CRISPR	HDR-mediated correction of mutation in mouse zygote
Cystic fibrosis	CRISPR	HDR-mediated correction of CFTR in intestinal stem cell organoid
Hereditary tyrosinemia	CRISPR	HDR-mediated correction of mutation in liver

6.1.7.1 Other CRISPR Vehicles

In Nature (Ran et al 2015) we have an article demonstrating a variant on the now standard CRISPR cas9 vehicle. As they first note:

Type II CRISPR-Cas systems require only two main components for eukaryotic genome editing: a Cas9 enzyme, and a chimeric sgRNA derived from the CRISPR RNA (crRNA) and the noncoding trans-activating crRNA (tracrRNA). Analysis of over 600 Cas9 orthologues shows that these enzymes are clustered into two length groups with characteristic protein sizes of approximately 1,350 and 1,000 amino acid residues, respectively

Thus the classic source is Streptococcus pyogenes and as noted:

The RNA-guided endonuclease Cas9 has emerged as a versatile genome-editing platform. However, the size of the commonly used Cas9 from Streptococcus pyogenes (SpCas9) limits its utility for basic research and therapeutic applications that use the highly versatile adeno-associated virus (AAV) delivery vehicle.

But the same vehicle with a Cas9 is in many other bacteria and they note:

Here, we characterize six smaller Cas9 orthologues and show that Cas9 from Staphylococcus aureus (SaCas9) can edit the genome with efficiencies similar to those of SpCas9, while being more than 1 kilobase shorter. We packaged SaCas9 and its single guide RNA expression cassette into a single AAV vector and targeted the cholesterol regulatory gene Pcsk9 in the mouse liver.

Thus we have a variant but the same functionality. They conclude regarding in vivo changes:

Here, we develop a small and efficient Cas9 from S. aureus for in vivo genome editing. The results of these experiments highlight the power of using comparative genomic analysis in expanding the CRISPR-Cas9 toolbox. Identification of new Cas9 orthologues, in addition to structure-guided engineering, could yield a repertoire of Cas9 variants with expanded capabilities and minimized molecular weight, for nucleic acid manipulation to further advance genome and epigenome engineering. ...

We examined these sites in liver tissue transduced by AAV-SaCas9 and did not observe any indel formation within the detection limits of in vitro BLESS and targeted deep sequencing. Importantly, the off-target sites identified in vitro might differ from those in vivo, which need to be further evaluated by the applications of BLESS or other unbiased techniques such as those published during the revision of this work. Finally, we did not observe any overt signs of acute toxicity in mice at one to four weeks after virus administration. ....these findings suggest that in vivo genome editing using SaCas9 has the potential to be highly efficient and specific.

This is an interesting next step.

6.1.7.2 CRISPR Concerns

The system used by bacteria to defend against a virus attacking is the CRISPR Cas 9 system. An interesting use of a protein, enzyme, and a DNA segment that can open DNA at desired locations and cut and insert new segments of DNA. We have been discussing this for well over a year now and have discussed its potential and its risks.

Now along comes researchers who instead of doing this in somatic cells do it in germline cells, thus changing the potentially maturing entity. Thus each cell has this changed gene or genes.

In a recent Nature article the authors state:

There are grave concerns regarding the ethical and safety implications of this research. There is also fear of the negative impact it could have on important work involving the use of genome-editing techniques in somatic (non-reproductive) cells....In our view, genome editing in human embryos using current technologies could have unpredictable effects on future generations. This makes it dangerous and ethically unacceptable. Such research could be exploited for non-therapeutic modifications. We are concerned that a public outcry about such an ethical breach could hinder a promising area of therapeutic development, namely making genetic changes that cannot be inherited. At this early stage, scientists should agree not to modify the DNA of human reproductive cells. Should a truly compelling case ever arise for the therapeutic benefit of germ-line modification, we encourage an open discussion around the appropriate course of action.

Now this point is well made. Germline cell changes introduce all sorts of issues. Not only is there the issue of what this new gene will do, we hardly have begun to understand gene interactions, but the issues of epigenetic factors such as methylation dramatically change the risks.

Frankly I miss Michael Circhton, in this case he would have clearly shown us the mistakes we could be making with an unruly unleashing of this technology. Jurassic Park would be a walk in the park as compared to what these could unleash. Imagine correcting those few genes in Apes and the other close to man mammals and see what we could get!

The again you do have the advocates in Technology Review, that somewhat unidentifiable magazine sent to MIT alumni and others, that states:

When I visited the lab last June, ... proposed that I speak to a young postdoctoral scientist named ..., a Harvard recruit from Beijing who'd been a key player in developing a new, powerful technology for editing DNA, called CRISPR-Cas9. With ..., ...had founded a small company to engineer the genomes of pigs and cattle, sliding in beneficial genes and editing away bad ones. As I listened to ..., I waited for a chance to ask my real questions: Can any of this be done to human beings? Can we improve the human gene pool? The position of much of mainstream science has been that such meddling would be unsafe, irresponsible, and even impossible. But ... didn't hesitate. Yes, of course, she said. In fact, the Harvard laboratory had a project to determine how it could be achieved.

She flipped open her laptop to a PowerPoint slide titled "Germline Editing Meeting." Here it was: a technical proposal to alter human heredity. "Germ line" is biologists' jargon for the egg and sperm, which combine to form an embryo. By editing the DNA of these cells or the embryo itself, it could be possible to correct disease genes and to pass those genetic fixes on to future generations. Such a technology could be used to rid families of scourges like cystic fibrosis. It might also be possible to install genes that offer lifelong protection against infection, Alzheimer's, and, ... told me, maybe the effects of aging. These would be history-making medical advances that could be as important to this century as vaccines were to the last.

The problem is, as the writers in Nature and in Science noted, led by David Baltimore, have noted, the germ line modifications could be unwieldy.

Just because we have a new technology is no reason to let is loose. The problem with this technology is that it not only can be weaponized but that it can be done in a basement lab. This not the building of a nuclear weapon. This is potentially setting the world afire.

The again there is the issue of Government regulation. In an interesting piece in Xconomy the author remarks:

But researchers' and investors' fear that a patchwork of regulation would cripple biotechnology in the United States did not disappear right away. Biologist Thomas Maniatis of Harvard left his home lab to work on the techniques in tighter-security conditions at Cold Spring Harbor Laboratory in New York. Others went abroad. Biogen, founded in 1978, put its first major lab in Geneva, Switzerland. This was a time of intense concern about environmental dangers from the chemical industry in particular and science in general. It took some years for biologists to gain respect among local state, and federal officials for their sense of responsibility in the recombinant DNA maelstrom of the mid-1970s. But politicians did accept that biotechnology

was a significant new industry that other countries, like Japan, might seize if America dropped the ball.

A valid point, but in the 70s we worried about errant scientists. Now we are terrified about terrorist post docs! One wonders what would be worse; the Government Regulators or the Terrorist?

CRISPR Cas 9 is a new technique to cut and splice genes. We had written about it about a year ago regarding its use in cancer treatment and also regarding the patent so quickly issues. Now David Baltimore, a highly respected scientist, and colleagues have in Science suggested a prudent set of steps as to its use in humans. It is reminiscent of the concerns some 49 years ago regarding recombinant DNA.

Baltimore et al recommend:

In the near term, we recommend that steps be taken to:

1) Strongly discourage, even in those countries with lax jurisdictions where it might be permitted, any attempts at germline genome modification for clinical application in humans, while societal, environmental, and ethical implications of such activity are discussed among scientific and governmental organizations. (In countries with a highly developed bioscience capacity, germline genome modification in humans is currently illegal or tightly regulated.) This will enable pathways to responsible uses of this technology, if any, to be identified.

2) Create forums in which experts from the scientific and bioethics communities can provide information and education about this new era of human biology, the issues accompanying the risks and rewards of using such powerful technology for a wide variety of applications including the potential to treat or cure human genetic disease, and the attendant ethical, social, and legal implications of genome modification.

3) Encourage and support transparent research to evaluate the efficacy and specificity of CRISPR-Cas9 genome engineering technology in human and nonhuman model systems relevant to its potential applications for germline gene therapy. Such research is essential to inform deliberations about what clinical applications, if any, might in the future be deemed permissible.

4) Convene a globally representative group of developers and users of genome engineering technology and experts in genetics, law, and bioethics, as well as members of the scientific community, the public, and relevant government agencies and interest groups—to further consider these important issues, and where appropriate, recommend policies.

Baltimore et al have a point. Not only can this be significant on a person by person basis but it also has the potential to be weaponized. The technology is out there, thousands are now proficient in it, the cost is low and the means for distribution is high.

Clearly a sensible effort in collaboration with others is essential. The problem is that with much of science, the genie is out of the box.

6.1.7.3 Issue of Concern

As noted there may be many unintended consequences which have yet to be explored. We examine a few of them here and we are certain many more will arise in the future. This is a powerful technology, one that is inexpensive and fast acting, and one which if in th wrong hands can be used for less than benign purposes.

6.1.7.3.1 Accuracy of Cutting

How accurate are these breaks? There are two elements. One is the actual targeting and that seem to be excellent. The other would be the equivalent of a false targeting. Namely targeting an identical string at the wrong place.

6.1.7.3.2 Promoters and Other Interactions

We know that just having the right gene does not mean that it is expressed. Thus promoters and similar interactions must be considered. Gene expression is oftentimes a complicated process.

6.1.7.4 Methylation Factors

Methylation is well known to play active roles in gene expression. In the cut and paste mode we may change methylation profiles. This could dramatically change gene expression, since one gene product may be another gene promoter.

6.1.8 Other Epigenetic Factors

There are many other epigenetic factors including acetylation, miRNA, lncRNAs and the like. It is uncertain how these factors can be influenced in this process.

6.1.9 Some Questions

The CRISPR-Cas9 system has proven to be a workable in vivo editing mechanism for specific gene cut and paste situations. However there are several key questions that seem to hang over it. None are so severe as to cause substantial concern but in toto they clearly indicate potential but substantial work is still required, especially in the area of cancer therapeutics.

We thus present and discuss several such questions:

1. Can the CRISPR-Cas9 system target the correct sets of aberrant cancer genes?

The issue here is that in many cancers we have a multiple set of genes which are aberrant. To make it even more complex, there are cancer cells with different mixtures of mutated or inoperable genes. How thus does one target this broad and varying complex. A single genetic mutation is one thing an broad complex set of changes is another.

2. Can CRISPER-Cas9 system be delivered in vivo in a more effective manner?

The current delivery mechanism is targeted at specific cells in a specific location. What does one do with a metastatic cancer. Oftentimes you do not even know where the cells may be. Then again one also faces the issue of the stem cell and it special characteristic. This delivery most likely be difficult.

3. Is CRISPR-Cas9 a dose related system approach rather than an all-encompassing curative approach? Namely does it cut-and-paste a large set of genes but perhaps not all?

Is the delivery system akin to dosing in normal pharmacokinetics or is it a totally different mechanism.

4. How does CRISPR-Cas deal with metastatic cells wherein there are multiple sets of genetic alterations?

The multiplicity of gene breakdowns and the process in which this happens becomes a complex driver for applying this technology. Is there a single key to solving the problem or must one continue to track changes and chase the shadows of the genetic changes?

5. What are the potential deleterious sequellae possible from a CRISPR approach and how can they best be avoided?

The ultimate question will be what else this process can do. The unintended consequences may be significant. As was noted above:

That doesn't mean CRISPR is perfect, though. While it's extremely precise, it occasionally modifies DNA at similar sites elsewhere in the genome instead of the target gene.

What then are those mistakes which can occur, especially when targeting multiple genes?

6.1.10 Appendix: Terms and Definitions

Term	*Definition*
Cas9[27]	CRISPR-associated (Cas) proteins to direct degradation of complementary sequences present within invading viral and plasmid DNA. The *Streptococcus pyogenes* SF370 type II CRISPR locus consists of four genes, including the Cas9 nuclease, as well as two noncoding CRISPR RNAs (crRNAs): trans-activating crRNA (tracrRNA) and a precursor crRNA (pre-crRNA) array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (DRs)
CRISPR	Clustered regularly interspaced short palindromic repeats.
crRNA	CRISPR RNA

[27] Jinek et al

Term	Definition
tracrRNA[28]	In contrast, type II systems process precrRNAs by a different mechanism in which a ***trans-activating crRNA (tracrRNA) complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded*** (ds) NAspecific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein
sgRNA[29]	Type II CRISPR–Cas systems have been adapted as a genome-engineering tool. In this system, crRNA teams up with a second RNA, called trans-acting CRISPR RNA (tracrRNA), which is critical for crRNA maturation and recruiting the Cas9 nuclease to DNA. The RNA that guides Cas9 uses a short (20-nt) sequence to identify its genomic target. ***This three-component system was simplified by fusing together crRNA and tracrRNA, creating a single chimeric ''guide'' RNA abbreviated as sgRNA or simply gRNA.***
PAM[30]	The CRISPR locus is transcribed and processed into short CRISPR RNAs (crRNAs) that guide the Cas to the complementary genomic target sequence. There are at least eleven different CRISPR– Cas systems, which have been grouped into three major types (I–III). In the type I and II systems, nucleotides adjacent to the protospacer in the targeted genome comprise the protospacer adjacent motif (PAM). The PAM is essential for Cas to cleave its target DNA, enabling the CRISPR–Cas system to differentiate between the invading viral genome and the CRISPR locus in the host genome, which does not incorporate the PAM. For additional details on this fascinating prokaryotic adaptive immune response.
Homologous	Homologous chromosomes are a set of one maternal chromosome and one paternal chromosome that pair up with each other inside a cell during meiosis.
RuvC[31]	RuvC is the resolvase, which cleaves the Holliday junction. It is thought to bind either on the open, DNA exposed face of a single RuvA tetramer, or to replace one of the two tetramers. Binding is proposed to be mediated by an unstructured loop on RuvC, which becomes structured on binding RuvA. RuvC can be bound to the complex in either orientation, therefore resolving Holliday junctions in either a horizontal or vertical manner. Cas9 contains domains homologous to both HNH and RuvC endonucleases.
HNH[32]	The domain HNHc (SMART ID: SM00507, SCOP nomenclature: HNH family) is associated with a range of DNAbinding proteins, performing a variety of binding and cutting functions. Several of the proteins are hypothetical or putative

[28] Jinek et al
[29] Harrison et al
[30] Harrison et al
[31] Bennett and West
[32] Mehta et al

Term	Definition
	proteins of no well-defined function. The ones with known function are involved in a range of cellular processes including bacterial toxicity, homing functions in groups I and II introns and inteins, recombination, developmentally controlled DNA rearrangement, phage packaging, and restriction endonuclease activity Cas9 contains domains homologous to both HNH and RuvC endonucleases

7 GENE DRIVE

The current capabilities of gene manipulation, also we can probably call it gene engineering, is that we have an ever growing collection of "tools". These tools allow bench folks to add this or subtract that from a cell. We can do this with a somatic cell, an existing cell in an existing organ and a germ line cell, a cell from which the organism will eventually be derived. We can add or delete genes and we can in so doing insert a mechanism which will carry on this editing process no matter what the cell becomes as the organism develops. Furthermore if we get this process working in the germ line cell then we can be assured that as the next generation proceeds this inserted tool for manipulating genes within the organism, if not now within the total species, proceeds in a dramatic manner. We lose Mendelian genetics and the tool insertion now produces a single unaltered lineage.

If this gene is of a certain type, say one which produces only a male, then by blocking in all subsequent lines of any females we can effectively wipe out this species when we have just surviving but non-producing males.

This assembly of tools by the genetic engineer has been called "gene drives". In a sense it "drives" certain genes into all members of a species. At least that is the hope. As the Broad Institute states in its licensing statements[33]:

Gene drive. This is a way to rapidly spread a new gene throughout an entire species in nature. This approach might be used to block the transmission of malaria by mosquitoes, but has the potential to disrupt ecosystems… After consulting with external experts and careful internal consideration, the Broad Institute has decided to make available non-exclusive research and commercial licenses for the use of CRISPR technology in agriculture -- but with important restrictions. These include: Gene drive: We prohibit the use of the licensed technology for gene drive.

7.1.1 The Principle

We briefly present the principles in a somewhat simplistic fashion. Yet the overall idea is also somewhat straightforward. Namely, it is the ability to utilize the set of gene manipulation tools in such a manner as to achieve dramatic results.

Let us begin with some comments made in early 2015 by Bohannon who notes:

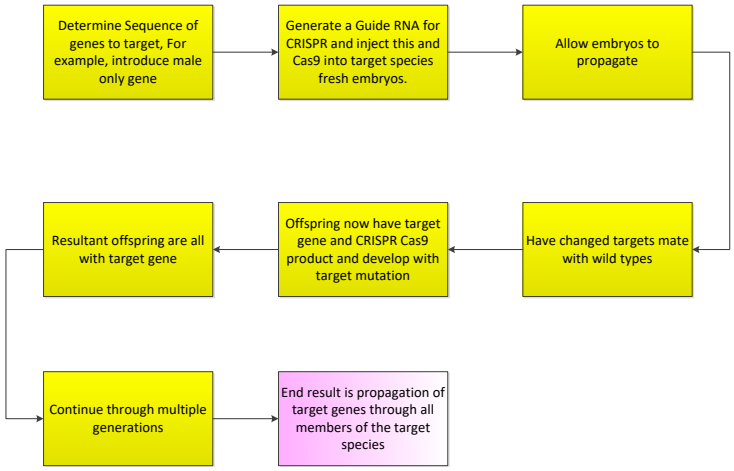
In 28 December 2014, Valentino Gantz and Ethan Bier checked on the fruit flies that had just hatched in their lab at the University of California (UC), San Diego. By the classic rules of Mendelian genetics, only one out of four of the newborn flies should have shown the effects of the mutation their mothers carried, an X-linked recessive trait that causes a loss of pigmentation similar to albinism.

[33] https://www.broadinstitute.org/news/licensing-crispr-agriculture-policy-considerations

Instead, nothing but pale yellow flies kept emerging. "We were stunned," says Bier, who is Gantz's Ph.D. adviser. "It was like the sun rose in the west rather than the east." They hammered out a paper and submitted it to Science 3 days later….Gantz and Bier report that the introduced mutation disabled both normal copies of a pigmentation gene on the fruit fly chromosomes, transmitting itself to the next generation with 97% efficiency—a near-complete invasion of the genome. The secret of its success: an increasingly popular gene-editing toolkit called CRISPR (Science, 23 August 2013, p. 833), which Gantz and Bier adapted to give the mutation an overwhelming advantage.

The technique is the latest—and some say, most impressive—example of gene drive: biasing inheritance to spread a gene rapidly through a population, or even an entire species. At this level of efficiency, a single mosquito equipped with a parasite-blocking gene could in theory spread malaria resistance through an entire breeding population in a single season

Now this principle can be depicted as shown below.



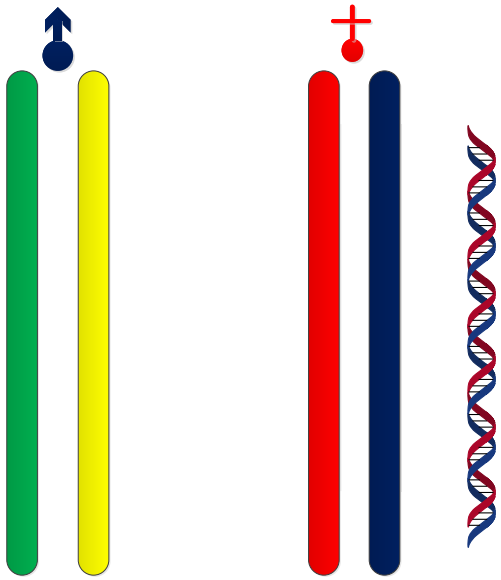
Specifically, the steps are simplified as follows:

1. Select Target Embryo
    a. Embryos are essential since we need to start with a pluripotent cell
2. Select Gene
    a. This is the key step. It is essential to know the target gene and its suppressive character
3. Select Vector
    a. This is a selection of an insert mechanism into the DNA of the embryo such as a lentivirus and reverse transcriptase
4. Select CRISPR
5. Select Cas9
    a. Cas9 is now but one of several endonucleases to cut DNA using the designed CRISPR
    b. One may seek to use others for better performance
6. Insert Gene for Change
7. Insert CRISPR/Cas9

8. Allow Embryo Maturation
9. Release Target Entity

We now cover the process in some high level detail.

Consider a male and female set of chromosome pairs as shown below. Each chromosome pair has one from each of its parents. Thus we depict four different DNA elements, each DNA a double strand. Nothing new here.



Now one question we may ask is if one of the chromosomes from a parent has a gene that results in a certain characteristic then how is it inherited downstream? Simply one can use classic Mendelian genetics and create squares and even include linkages. If but one parent has one gene change then we would expect say one fourth, the off-spring to have that trait. Furthermore, if that offspring were to mate a wild type, namely one without that gene, then we would see the same. Actually the gene would be diluted in the total population.

Thus the question we pose is twofold:

1. How do we insert a gene which can control offspring, perhaps to the disadvantage of the species?

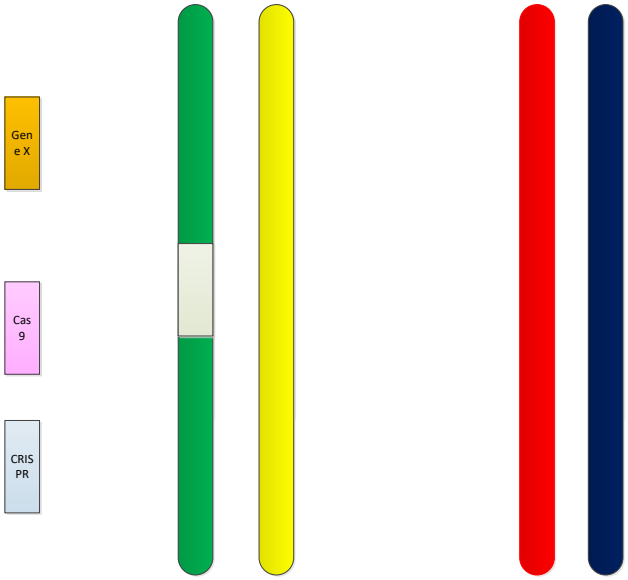
2. How can we alter the inheritance so that every subsequent off-spring will inherit the desired gene to the disadvantage of the species? This is the concept of driving genes into a species.

The answer is twofold:

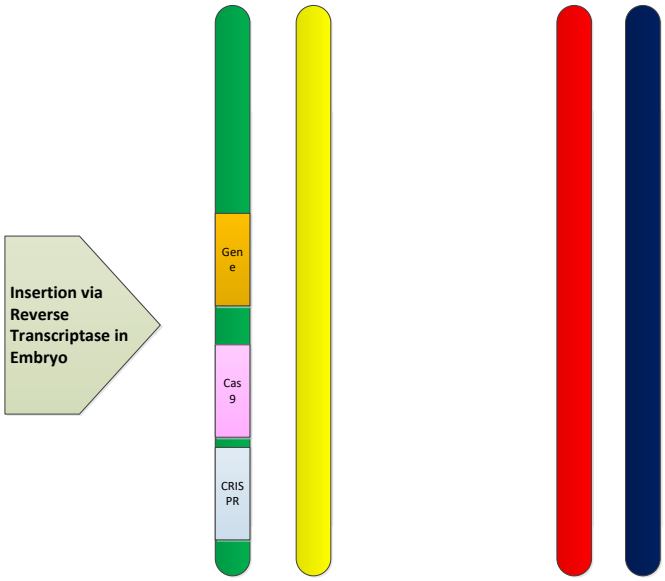
1. First we can insert the gene into an embryo via now standard gene insertion mechanisms such as lentivirus and reverse transcriptase.

2. Second, we can also insert a CRISPR to target the gene propagation and a Cas9 to cut other genes for that insertion so that we have a mechanism to cut and past the desired gene in all other chromosomes, including the wild type mating. We thus create a self-replicating gene insertion so that all off-spring will have every one of their chromosomes get an inserted gene.

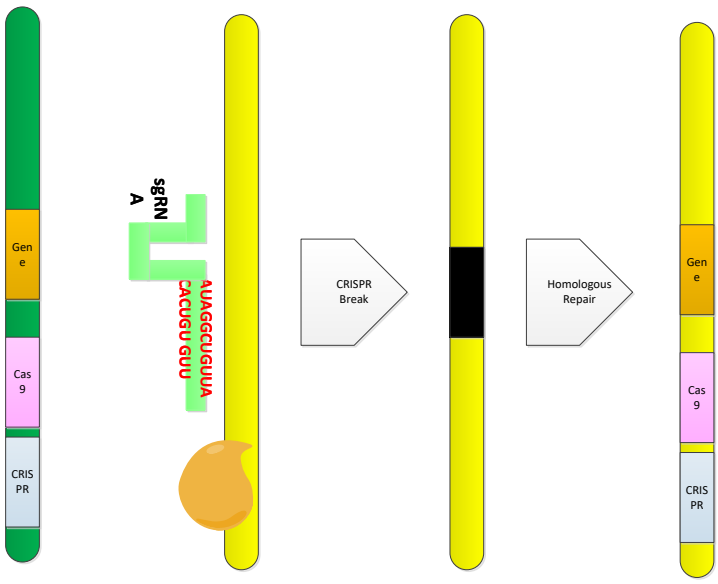
The process begins as below where we want to insert these three segments.



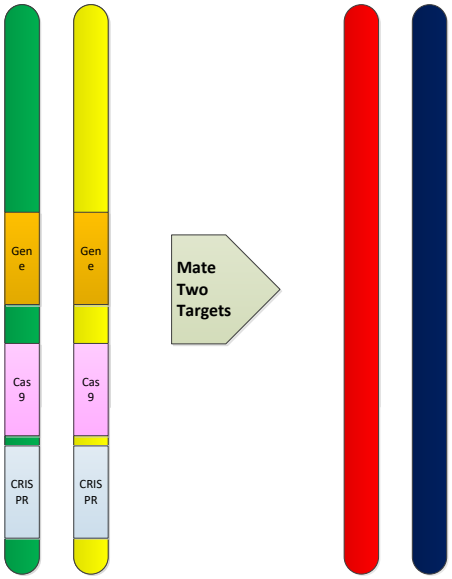
The we can proceed with the insertion of an embryo before it begins to double. That is we find say a mosquito embryo and then proceed to insert the desired genes.



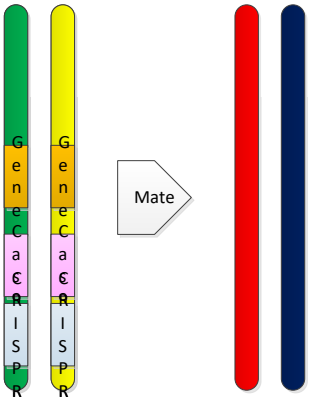
Then the CRISPR machine starts working to insert itself everywhere.

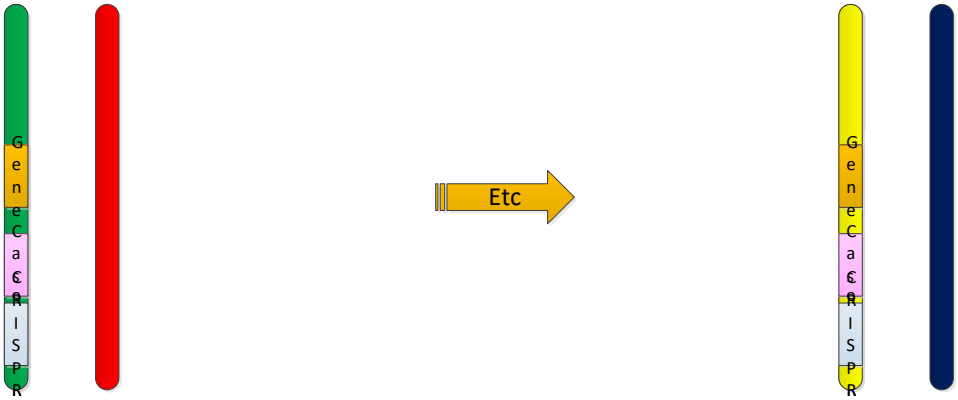


Then we mate this changed vector to a wild type target as shown below.

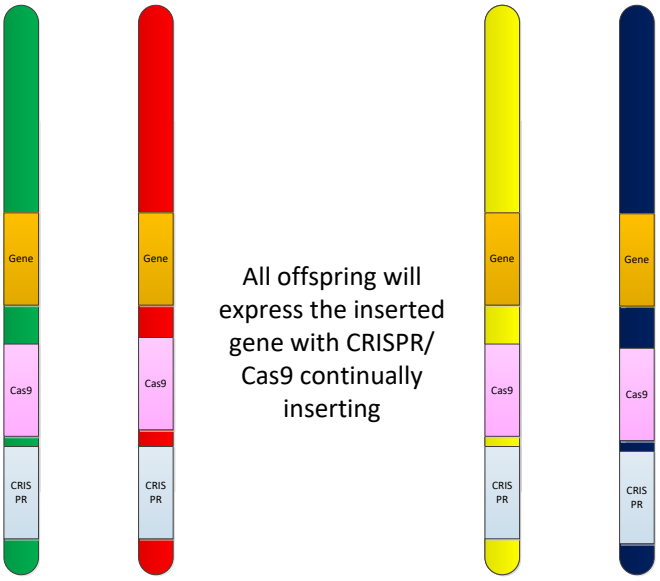


The result of the mating is as per below.





And the process is repeated again and again.



The net result in this simplistic process is the driving of the desired genes into the species by this self-replicating manner.

7.1.1.1 Underlying Elements

We will now review some of the details of the above summarized steps. Our approach is high level and does not reflect any specific bench process. As we have noted elsewhere the development of gene manipulation "tools" has progressed at a rapid rate. It is in a sense akin to the progress in Chemistry some 150 years ago, ways to distill, separate, purify, various compounds, and in the gene space the "tools" often simple and yet to be fully appreciated, are just similar.

7.1.1.2 Outcome Selection

The first step is what we call outcome selection. What does one want to achieve and what can be achieved. Also, there is the issue of consequences. The process of Outcome Selection will most likely be controlled by some IRB process, akin to drug trials. In drug trials there is the possibility for human harm, but will this be the case with Gene Drives, in fact there could be massive human harm.

Thus, the process of Outcome Selection should be formalized, documented, reviewed and approved. The problem however is the ability for many of these Gene Drive procedures to be performed in random uncontrolled environment without any controls. That as we shall see is the most significant risk.

7.1.1.3 Gene Selection

Let us examine how the genes are selected. We focus first on the work of Sutton et al. They state:

In this study we have identified a set of genes with testis-specific expression or splicing. In addition to their interest from a basic biology perspective, these findings provide a basis from which to develop synthetic systems to control important pest insects via manipulation of the male germline.…Current strategies for insect control have a number of disadvantages, such as effects on non-target species and development of resistance to insecticides. Alternative synthetic biology approaches are being developed in which the control agent is a modified version of the pest insect itself. These modified insects carry a genetic system that results in the death of some or all of their descendants, so that when released modified insects mate with wild counterparts, population suppression occurs.

Such strategies require characterized modular components that can direct appropriate expression of effector sequences – protein-coding sequences or functional RNAs, for example. Conserved components that can be used across multiple species are particularly useful. However, for many applications there are few if any such components available. The goal of this study was to identify genes that could provide potential components for manipulation of the male

germline in two major pest species, the mosquito Aedes aegypti (L.) and the tephritid fruit fly Ceratitis capitate…

High-throughput transcriptional profiling and subtractive hybridization studies have recently yielded several potential testis-specific transcripts in Ae. aegypti. However, to our knowledge, no studies have been performed with sufficient time resolution to determine the activity of regulatory regions at different stages of spermatogenesis. Information on insect testis-specific splicing is even more sparse; testis-specific splice forms of the genes achi and vis have been discovered in D. melanogaster, but no testis-specific splice forms have been identified, to our knowledge, in Ae. aegypti, C. capitata or any other pest insect.

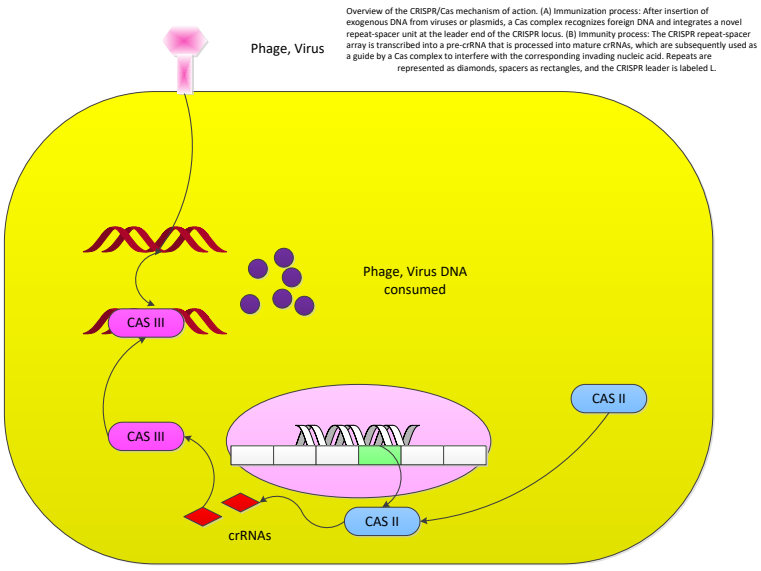
Thus there are now a multiplicity of ways to control such species. The above reduces the offspring to all male. There clearly are many others.

7.1.1.4 Embryo

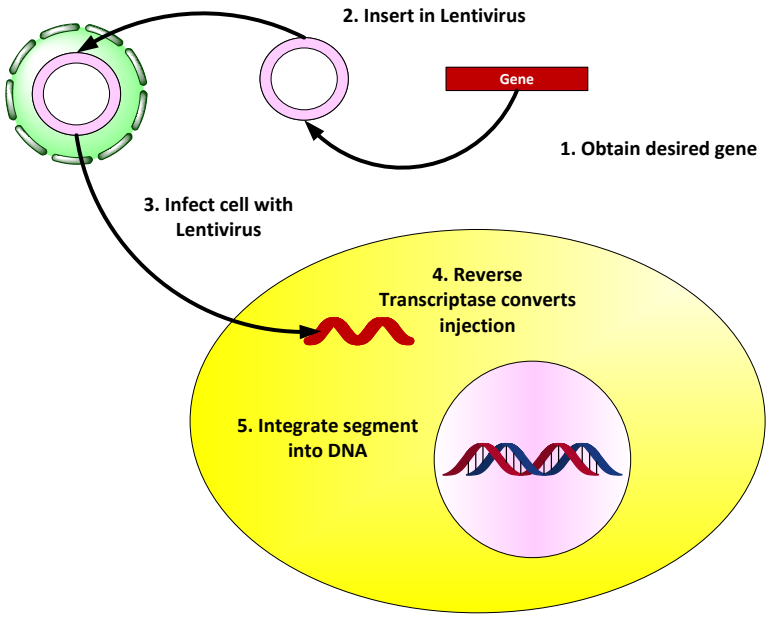
We start typically with an embryo. This is the pluripotent cell, easily targeted say in an insect population. Likewise, in plants since all cells are pluripotent we can reasonably start anywhere.

7.1.1.5 Insertion

Insertion of the genes is performed in a standard manner. We have discussed these in our work on CAR T Cells and refer the reader to those details. We show below a simplified method of insertion using a phage and an effecting it through a reverse transcriptase. This is one of the first of the many tools we see applied here.



The above demonstrates how this applies in the context of Car insertions and the diagram depicts the lentivirus approach from the CAR T cell environment. In effect one can "infect" the embryo with the desired gene segments, get them inserted using the reverse transcriptase and have this done on one or even both chromosomes.



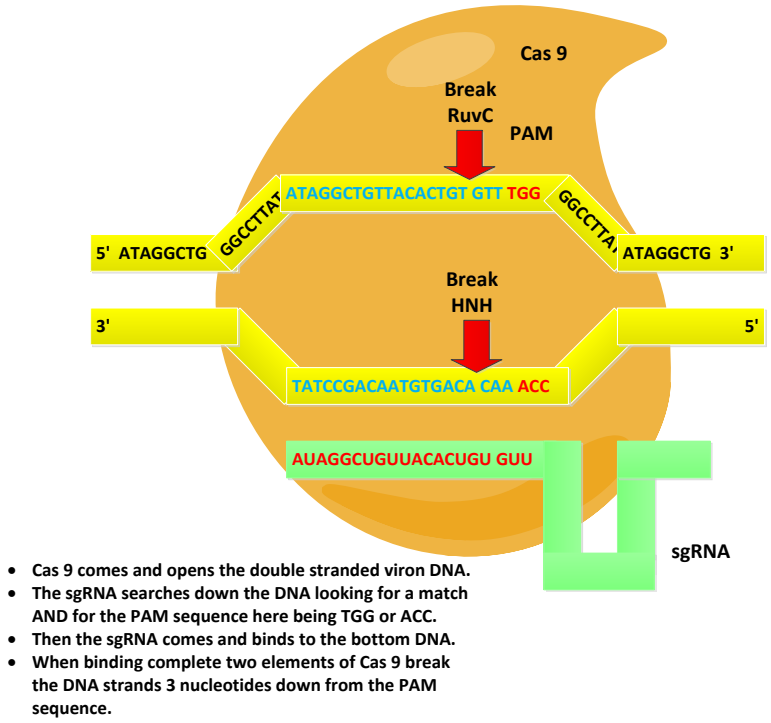
7.1.1.6 Execution

We now move on towards the execution. We first consider the CRISPR issues.

7.1.1.7 CRISPR Cas9 System

CRISPR is an RNA guide that is used in conjunction with the Cas9 endonuclease. Simply CRISPR targets a location to cut and Cas9 does a double side break of the DNA. Then the new gene is inserted and the break repaired by a DNA repair mechanism. We details this in our report on CRISPRs and Cancer, see references.

We demonstrate the CRISPR/Cas9 system below.



7.1.1.8 Cpf1 System

We have examined CRISPRs for the past few years since their introduction. Initially we had a CRISPR with a Cas9 molecule which managed to cut DNA at specific spots. The CRISPR was designed to match a specific sequence and the Cas9 was able to recognize the PAM sequences and using certain portions of the Cas9 it could then "break" both strands at opposite positions of the DNA, a specific set of base pairs from the end of the PAM.

This then becomes a useful tool in an ever-growing tool-box for DNA modification. In bacteria this cut is applied to viral DNA or RNA and it is a "natural" immune system in the bacteria. In other cells, plants and animals, it enables precise and specific gene editing.

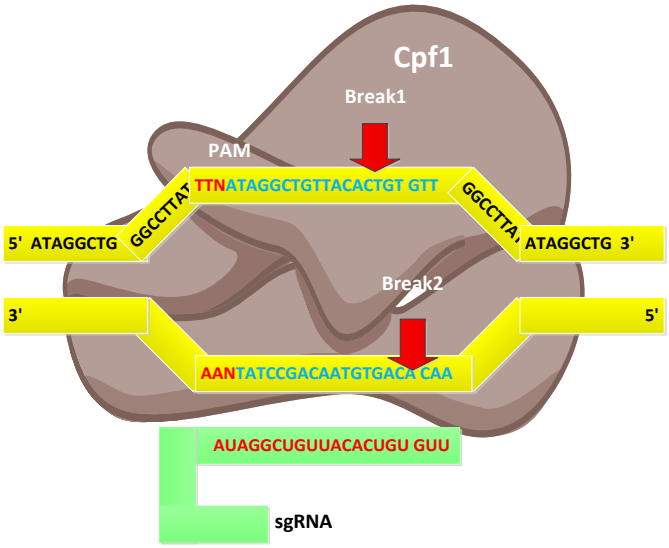
In a recent paper from Zhang's Lab at Broad they have identified another protein which acts like Cas9. This new system is called CRISPR-Cpf1 and is identified as a class 2 CRISPR system[34] . Specifically Cpf1 is a CRISPR-associated two-component RNA-programmable DNA nuclease. It functions in a manner similar to Cas9 and targeted DNA is cleaved as a 5-nt staggered cut distal to a 5′ T-rich PAM. They have also identified two Cpf1 orthologs exhibit robust nuclease activity in human cells. In the paper in Cell they state:

The microbial adaptive immune system CRISPR mediates defense against foreign genetic elements through two classes of RNA-guided nuclease effectors. Class 1 effectors utilize multi-

[34] http://www.cell.com/cell/abstract/S0092-8674%2815%2901200-3

protein complexes, whereas class 2 effectors rely on single-component effector proteins such as the well-characterized Cas9. Here, we report characterization of Cpf1, a putative class 2 CRISPR effector. We demonstrate that Cpf1 mediates robust DNA interference with features distinct from Cas9. Cpf1 is a single RNA-guided endonuclease lacking tracrRNA, and it utilizes a T-rich protospacer-adjacent motif. Moreover, Cpf1 cleaves DNA via a staggered DNA double-stranded break. Out of 16 Cpf1-family proteins, we identified two candidate enzymes from Acidominococcus and Lachnospiraceae, with efficient genome-editing activity in human cells. Identifying this mechanism of interference broadens our understanding of CRISPR-Cas systems and advances their genome editing applications.

The figure below depicts their interpretation of its functioning.



- *Cpf1 system is simpler than Cas9 in that it requires only a single RNA. The Cpf1 enzyme is also smaller than the standard SpCas9, making it easier to deliver into cells and tissues.*
- *Cpf1 cuts DNA in a different manner than Cas9. When the Cas9 complex cuts DNA, it cuts both strands at the same place, leaving "blunt ends" that often undergo mutations as they are rejoined. With the Cpf1 complex the cuts in the two strands are offset, leaving short overhangs on the exposed ends.*
- *Cpf1 cuts far away from the recognition site, meaning that even if the targeted gene becomes mutated at the cut site, it can likely still be recut, allowing multiple opportunities for correct editing to occur.*
- *Cpf1 system provides new flexibility in choosing target sites. Cpf1 complex must first attach to a short sequence known as a PAM, and targets must be chosen that are adjacent to naturally occurring PAM sequences.*
- *Cpf1 complex recognizes very different PAM sequences from those of Cas9. This could be an advantage in targeting some genomes, such as in the malaria parasite as well as in humans.*

It is worth comparing these two mechanisms. The Cas9 is a bit more rigid than Cpf1. As noted above and as discussed in the paper and elsewhere, this new protein complex does what Cas9 did but with many more attractive features.

In an MIT press release they state[35] :

The newly described Cpf1 system differs in several important ways from the previously described Cas9, with significant implications for research and therapeutics, as well as for business and intellectual property:

[35] http://mcgovern.mit.edu/news/news/system-for-genome-editing-could-increase-power-of-genome-engineering/

First: In its natural form, the DNA-cutting enzyme Cas9 forms a complex with two small RNAs, both of which are required for the cutting activity. The Cpf1 system is simpler in that it requires only a single RNA. The Cpf1 enzyme is also smaller than the standard SpCas9, making it easier to deliver into cells and tissues.

Second, and perhaps most significantly: Cpf1 cuts DNA in a different manner than Cas9. When the Cas9 complex cuts DNA, it cuts both strands at the same place, leaving "blunt ends" that often undergo mutations as they are rejoined. With the Cpf1 complex the cuts in the two strands are offset, leaving short overhangs on the exposed ends. This is expected to help with precise insertion, allowing researchers to integrate a piece of DNA more efficiently and accurately.

Third: Cpf1 cuts far away from the recognition site, meaning that even if the targeted gene becomes mutated at the cut site, it can likely still be recut, allowing multiple opportunities for correct editing to occur.

Fourth: The Cpf1 system provides new flexibility in choosing target sites. Like Cas9, the Cpf1 complex must first attach to a short sequence known as a PAM, and targets must be chosen that are adjacent to naturally occurring PAM sequences. The Cpf1 complex recognizes very different PAM sequences from those of Cas9. This could be an advantage in targeting some genomes, such as in the malaria parasite as well as in humans.

The above four properties are quite compelling and worthy of note. Cas9 did have the problem of cutting at opposite sites and trusting that a competent and non-aberrant re-fusion was made. This discovery, assumedly after hundreds of attempts, opens the door on another dimension of the CRISPR world.

As is noted in Xcomony they state[36] :

… the Cpf1 work is still in its infancy. It's well behind CRISPR/Cas9—which researchers have used to make changes in the cells of all types of organisms, including humans. Several companies are working with CRISPR/Cas9 to create therapeutics for genetic disease. None have reached clinical trials yet.

The issue here is just how extensive is Cpf1 development and how readily available is the technology. The above presentation seems to imply an early stage. They continue:

But work with CRISPR/Cas9 to modify the human germline—eggs, sperm, and embryos—is also coming faster than expected, sparking ethical concerns. An international summit on the topic is scheduled for December in Washington, DC.

Meanwhile, researchers around the world are working to find new versions of Cas9, or new enzymes entirely, like Cpf1, to make the whole enterprise easier. "There is little doubt that… there are additional systems with distinctive characteristics that await exploration and could

[36] http://www.xconomy.com/boston/2015/09/25/crispr-update-could-make-gene-edits-easier-discoverers-say/

further enhance genome editing and other areas of biotechnology as well as shed light on the evolution of these defense systems," Zhang (pictured above speaking at a 2014 Xconomy event) and his coauthors write in the Cell paper.

In other words, Cpf1 is the tip of the iceberg. I'll outline three differences between Cpf1 and Cas9 that the paper's authors have highlighted as potentially important for the field. First, for those unfamiliar with CRISPR and gene editing, it helps to think of these enzymes as molecular scissors. Bacteria use them in the wild to defend themselves against invading viruses, cutting up the viral RNA and storing the pieces in a kind of immune system memory bank.

It was only in recent years that the natural system has been modified and harnessed as a gene editing tool. The enzyme—a protein—and its guide—made from RNA—need to be sent into a cell (that's one difficult trick) and hit the right spot (that's another difficult trick).

The following is the Xconomy author's description. It is a restatement of what was in the MIT release but rephrases the key differences:

Here's why Zhang and his co-authors think Cpf1 could have advantages over Cas9:

—Cpf1 only uses one strand of RNA as a guide to reach its target gene. Cas9 uses two strands. A single-strand system might lead to simpler, cheaper designs and easier delivery of the enzyme-guide complex into cells.

—Once delivered into the cell's nucleus, Cpf1 makes staggered double-stranded cuts in the target DNA, whereas Cas9 cuts both DNA strands in the same location. This could be important, Zhang and colleagues write, because the staggered ends make it easier to insert a new gene after the old one is removed. That could help get around one of the hurdles of Cas9: Scientists say using Cas9 to replace an old gene with a new one has proven far more difficult than simply cutting out a gene.

—When Cpf1 homes in on a gene, it actually makes the cut off to the side, relatively speaking—farther down the DNA strand. (Imagine your friend holding a string in the exact location that needs snipping. You don't cut her finger; you cut off to the side.) Zhang and colleagues write that this could be a "potentially useful feature" because it preserves the target site for subsequent rounds of editing.

The off-setting of the splices is a significantly better method. It gives the "sticky" ends approach and tends to much fewer errors. This alone could make this much more attractive.

In a Nature discussion of these results they state[37] :

But now one of the technique's pioneers thinks that he has found a way to make CRISPR even simpler and more precise. In a paper published in Cell on 25 September, a team led by synthetic biologist Feng Zhang of the Broad Institute in Cambridge, Massachusetts, reports the discovery

[37] http://www.nature.com/news/alternative-crispr-system-could-improve-genome-editing-1.18432

of a protein1 called Cpf1 that may overcome one of CRISPR-Cas9's few limitations; although the system works well for disabling genes, it is often difficult to truly edit them by replacing one DNA sequence with another.

The CRISPR/Cas9 system evolved as a way for bacteria and archaea to defend themselves against invading viruses. It is found in a wide range of these organisms, and uses an enzyme called Cas9 to cut DNA at a site specified by 'guide' strands of RNA. Researchers have turned CRISPR/Cas9 into a molecular-biology powerhouse that can be used in other organisms. The cuts made by the enzyme are repaired by the cell's natural DNA-repair processes.
Good, better, best?

CRISPR is much simpler than previous gene-editing methods, but Zhang thought there was still room for improvement.

So he and his colleagues searched the bacterial kingdom to find an alternative to the Cas9 enzyme commonly used in laboratories. In April, they reported that they had discovered a smaller version of Cas9 in the bacterium Staphylococcus aureus2. The small size makes the enzyme easier to shuttle into mature cells — a crucial destination for some potential therapies.

The team was also intrigued by Cpf1, a protein that looks very different from Cas9, but is present in some bacteria with CRISPR. The scientists evaluated Cpf1 enzymes from 16 different bacteria, eventually finding two that could cut human DNA.

They also uncovered some curious differences between how Cpf1 and Cas9 work. Cas9 requires two RNA molecules to cut DNA; Cpf1 needs only one. The proteins also cut DNA at different places, offering researchers more options when selecting a site to edit. "This opens up a lot of possibilities for all the things we could not target before," says epigeneticist Luca Magnani of Imperial College London.

Cpf1 also cuts DNA in a different way. Cas9 cuts both strands in a DNA molecule at the same position, leaving behind what molecular biologists call 'blunt' ends. But Cpf1 leaves one strand longer than the other, creating a 'sticky' end. Blunt ends are not as easy to work with: a DNA sequence could be inserted in either end, for example, whereas a sticky end will only pair with a complementary sticky end.

"The sticky ends carry information that can direct the insertion of the DNA," says Zhang. "It makes the insertion much more controllable."

Zhang's team is now working to use these sticky ends to improve the frequency with which researchers can replace a natural DNA sequence. Cuts left by Cas9 tend to be repaired by sticking the two ends back together, in a relatively sloppy repair process that can leave errors. Although it is possible that the cell will instead insert a designated, new sequence at that site, that kind of repair occurs at a much lower frequency. Zhang hopes that the unique properties of how Cpf1 cuts may be harnessed to make such insertions more frequent.

In contrast, we also have an article in The Economist which states[38] :

CRISPR-Cpf1 may also be better than CRISPR-Cas9 in other ways. Cpf1 is a smaller and simpler enzyme (known technically as an endonuclease) than Cas9, which means it will be easier to deliver to the cells whose genes need modifying. And its slightly offset cuts to double-stranded DNA will help researchers to insert genetic patches more efficiently and accurately.

Its discovery also raises the question of how many other endonuclease-based systems are out there in the world's bacteria. Viral infection is a serious threat to these microbes, and the natural job of both CRISPR-Cas9 and CRISPR-Cpf1 is to recognize viral genes and chop them up before they can do any harm. Conversely, viruses are constantly evolving to escape the antiviral systems' attentions, meaning bacteria need to generate new ones. The chances are good, therefore, that CRISPR-Cas9 and CRISPR-Cpf1 are not alone. …

The tools to carry out that exploration now exist. CRISPR-Cpf1, for instance, was found not by searching in bacteria directly, but by scrutinizing a published database of bacterial genetic sequences, which yielded two species that contain it. Further searches might be equally rewarding—and the more gene-editing systems are discovered, the harder it will be to monopolies their use.

Despite the optimism of those who think the new techniques may calm qualms about genetic engineering, however, some people are bound to have ethical worries—certainly when it comes to applying them to human beings. Earlier this year, for example, when Chinese scientists used CRISPR-Cas9 gene editing on a human embryo (albeit one that was unviable, and could not therefore have developed into a person) there was much brouhaha and several calls for a moratorium on this line of inquiry.

There may not only be ethical worries but as we have discussed previously there is a weaponization approach also readily available. In a report in Nature World they state[39] :

The CRISPR/Cas9 system evolved as a way for bacteria and archaea to defend themselves against invading viruses. It is found in a wide range of these organisms, and uses an enzyme called Cas9 to cut DNA at a site specified by 'guide' strands of RNA. Researchers have turned CRISPR/Cas9 into a molecular-biology powerhouse that can be used in other organisms. The cuts made by the enzyme are repaired by the cell's natural DNA-repair processes…

The newly described Cpf1 system differs in several important ways from the previously described Cas9, with significant implications for research and therapeutics, as well as for business and intellectual property.

[38] http://www.economist.com/news/science-and-technology/21668031-scientists-have-found-yet-another-way-edit-genomes-suggesting-such-technology-will

[39] http://www.natureworldreport.com/2015/09/cpf1-for-precise-genome-engineering/

In its natural form, the DNA-cutting enzyme Cas9 forms a complex with two small RNAs, both of which are required for the cutting activity. The Cpf1 system is simpler in that it requires only a single RNA.

Cpf1 cuts DNA in a different manner than Cas9. When the Cas9 complex cuts DNA, it cuts both strands at the same place, leaving 'blunt ends' that often undergo mutations as they are rejoined. With the Cpf1 complex the cuts in the two strands are offset, leaving short overhangs on the exposed ends.

Cpf1 cuts far away from the recognition site, meaning that even if the targeted gene becomes mutated at the cut site, it can likely still be re-cut, allowing multiple opportunities for correct editing to occur.

The Cpf1 system provides new flexibility in choosing target sites. Like Cas9, the Cpf1 complex must first attach to a short sequence known as a PAM, and targets must be chosen that are adjacent to naturally occurring PAM sequences.

Finally, in a discussion in Wired the reporting is as follows[40] :

The discovery comes at a time when CRISPR/Cas9 is sweeping through biology labs. So revolutionary is this new genome editing technique that rival groups, who each claim to have been first to the tech, are bitterly fighting over the CRISPR/Cas9 patent. This new gene-editing protein called Cpf1—and maybe even others yet to be discovered—means that one patent may not be so powerful after all…

Many different proteins are associated with CRISPR. But in the early 2010s, Emmanuelle Charpentier, who was studying the flesh-eating bacteria Streptococcus pyogenes, stumbled onto one with special powers. Her bacteria happen to carry Cas9 proteins, which have the remarkable ability to precisely cut DNA based on a RNA guide sequence. In 2012, Charpentier and UC Berkeley biologist Jennifer Doudna published a paper describing the CRISPR/Cas9 system and speculated about its genome editing capabilities. And they filed a patent application. Much more on that patent later.

The patent issue is something we spoke about when the PTO pushed the Broad version through in less than six months, an unheard of process time.

While Cas9 has driven thousands of lab experiments and millions of dollars in funding for startups trying to capitalize on the technology, Cpf1 has remained relatively obscure. This study drags Cpf1 into the limelight. "It's a very comparable to Cas9 and it has a few different features which could be quite useful," says Dana Carroll, a biochemist at the University of Utah.

That's because Cas9 isn't perfect, despite its hype as a laser-precise genome editing tool. Cpf1 offers some slight advantages. For example, when it cuts double-stranded DNA, it snips the two strands in slightly different locations, resulting in overhang that molecular biologists call "sticky ends." Sticky ends can make it easier to insert a snippet of new DNA—say, a different version of

[40] http://www.wired.com/2015/09/war-genome-editing-just-got-lot-interesting/

a gene—though the Cell paper does not actually show data directly comparing Cas9 and Cpf1 when inserting DNA.

Cpf1 is also physically a smaller protein, so it may be easier to put into human cells. It requires only one RNA molecule instead of two, with Cas9. But it's not a rival so much as a complementary tool: The two proteins favor binding to different locations in the genome, so together, they might allow more flexibility in where scientist want to cut.

The writer then returns to the patent issues:

Not long after Doudna and UC Berkeley filed a patent, the Broad Institute and MIT filed their own patent on behalf of Zhang for the CRISPR/Cas9 system. Zhang had been working on actually showing that CRISPR/Cas9 can edit mammalian genomes in mammalian cells, an application he published in 2013 and says he came up with independently. The Broad's and MIT's attorney paid a fee to accelerate their application. Ultimately, the US Patent and Trademark Office awarded the patent to Zhang, MIT, and the Broad Institute. The University of California, obviously unhappy with the decision, filed an application for an interference proceeding to get the USPTO to reconsider. That process is ongoing.

But biotech companies have raced ahead to develop therapeutics and techniques with the system. Feng and Doudna have since licensed their technology to rival companies, Editas and Caribou. Charpentier also cofounded Crispr Therapeutics in Switzerland. Whoever wins the patent dispute will have a monopoly on CRISPR/Cas9 technology, the hottest new thing in biotech.

But with Cfp1, the stakes of that specific patent dispute go down. A lab or company could use Cfp1 without infringing on the CRISPR/Cas9 patent. "It takes power away from whoever the winner is going to be," says Jacob Sherkow, a NYU law professor. (Zhang has indicated the rights to Cpf1 may not necessarily go to the company he cofounded, Editas.) Whether a CRISPR/Cfp1 system is patentable as a separate invention—Sherkow says it probably is—perhaps isn't even relevant because its very existence means Cas9 is no longer the only game in town.

This latter observation is of significant value. Namely Cpf1 if it is truly better makes Cas9 battles of less value. It is of continuing interest to follow the dimensions of this new "tool box" available to those of us working on gene changes.

7.1.1.9 Other Endonucleases

The Scientist reports the identification of new enzymes to effect CRISPR targeting. Recall that CRISPR is a targeting RNA sequence and the enzyme, such as Cas9 is used to cut and then allow splicing of segments. CRISPR targets the gene position and the enzyme does the cutting. Cas9 does DSB or double stranded breaks. Other enzymes allow for sticky ends.

As The Scientist states[41]:

[41] http://www.the-scientist.com/?articles.view/articleNo/47845/title/New-CRISPR-Cas-Enzymes-Discovered/

Banfield's team searched the genomes for sequences that were both near cas1, which encodes a conserved CRISPR protein, and close to characteristic sequence repeats. The researchers found sequences for Cas9 in two archaeal genomes extracted from the Richmond Mine in Iron Mountain, California.

Previously, archaea were known to use class 1 CRISPR systems, but class 2 had only been identified in bacteria. "We don't really know how it performs, because that has not been achieved in the laboratory yet," said Banfield. "Archaea have different biology. The fact that [my collaborators] haven't yet managed to show its function probably means there are components of the system that we don't yet know about." The group also uncovered new types of Cas proteins from groundwater and soil bacteria, dubbed CasX and CasY. "They're really small, especially CasX," said Banfield. "That means it's potentially more useful."

CasX is made up of only 980 amino acids, whereas other Cas enzymes are larger. For instance, the commonly used Cas9 from Staphylococcus pyogenes contains 1,368 amino acids, while a smaller one from S. aureus is made up of 1,053 amino acids (CasY is around 1,200 amino acids). "This is important biotechnologically, because if you look at from the angle of genome editing, the delivery of small genes into cells is much easier than the delivery of large genes," ... In partnership with UC Berkeley's Jennifer Doudna, Banfield's team demonstrated that CasX and CasY are functional.

The researchers introduced CRISPR-CasX and CRISPR-CasY into E. coli, finding that they could block genetic material introduced into the cell.

The article mentioned above appears in Nature by Burnstein et al. The article states:

CRISPR-Cas systems provide microbes with adaptive immunity by employing short sequences, termed spacers, that guide Cas proteins to cleave foreign DNA. Class 2 CRISPR-Cas systems are streamlined versions in which a single Cas protein bound to RNA recognizes and cleaves targeted sequences. The programmable nature of these minimal systems has enabled their repurposing as a versatile technology that is broadly revolutionizing biological and clinical research.

However, current CRISPR-Cas technologies are based solely on systems from isolated bacteria, leaving untapped the vast majority of enzymes from organisms that have not been cultured. Metagenomics, the sequencing of DNA extracted from natural microbial communities, provides access to the genetic material of a huge array of uncultivated organisms. Here, using genome-resolved metagenomics, we identified novel CRISPR-Cas systems, including the first reported Cas9 in the archaeal domain of life.

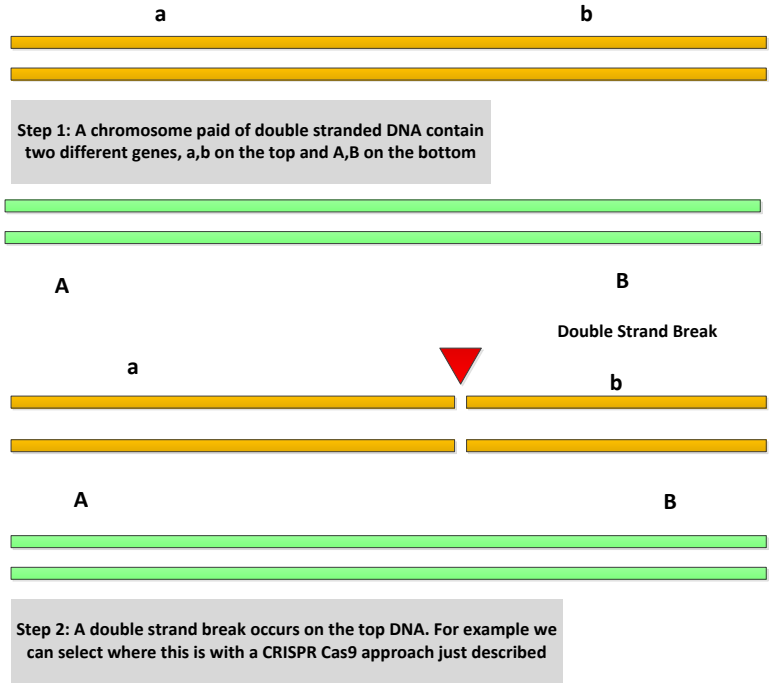
This divergent Cas9 protein was found in little-studied nanoarchaea as part of an active CRISPR-Cas system. In bacteria, we discovered two previously unknown systems, CRISPR-CasX and CRISPR-CasY, which are among the most compact systems yet identified. Notably, all required functional components were identified by metagenomics, enabling validation of robust in vivo RNA-guided DNA interference activity in E. coli. Interrogation of environmental

microbial communities combined with in vivo experiments allows access to an unprecedented diversity of genomes whose content will expand the repertoire of microbe-based biotechnologies.

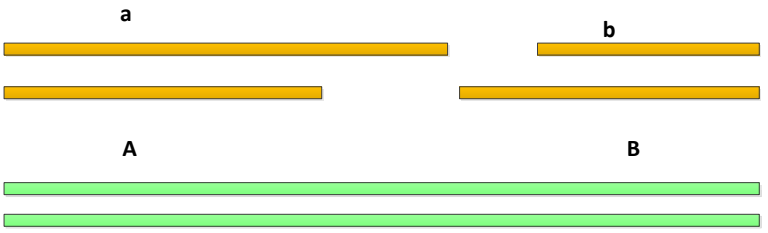
The targets and capabilities continue to expand.

7.1.1.10 Germ Line Inclusion

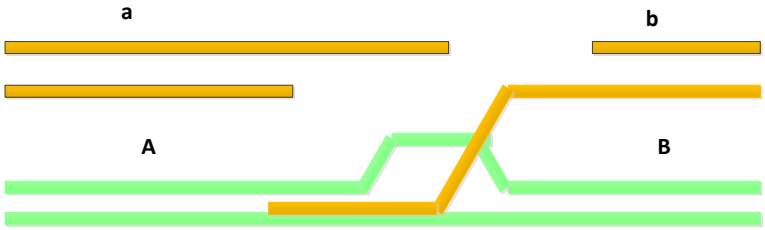
The following is a brief graphic summary of DSB repair. The first step the DS and then a single break of a single DS.



The next step is the shortening of one end and then the opening of the opposite chromosome DNA and the use of it for repair.

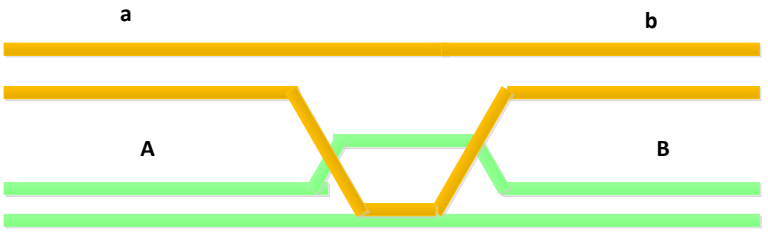


Step 3: The cut strands are shortened in top and bottom branches

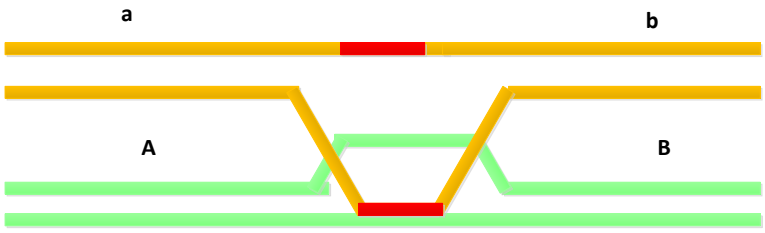


Step 4: The bottom branch uses the bottom branch as a renewal template reconstructing the full strand.

Finally the repair is achieved and the other end similarly repaired.



Step 5: Bottom completes and top finishes

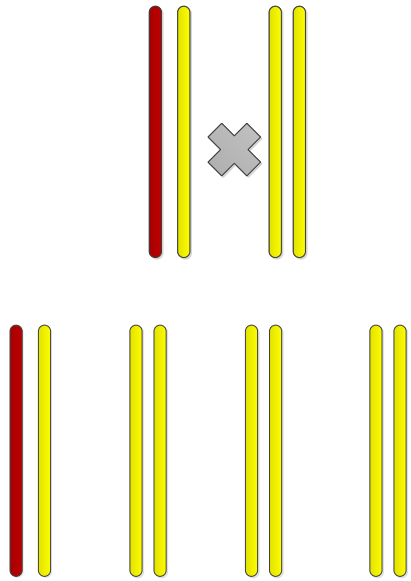


Step 6: A selected gene can be inserted and added, or it can equally well be removed by a two cut process.

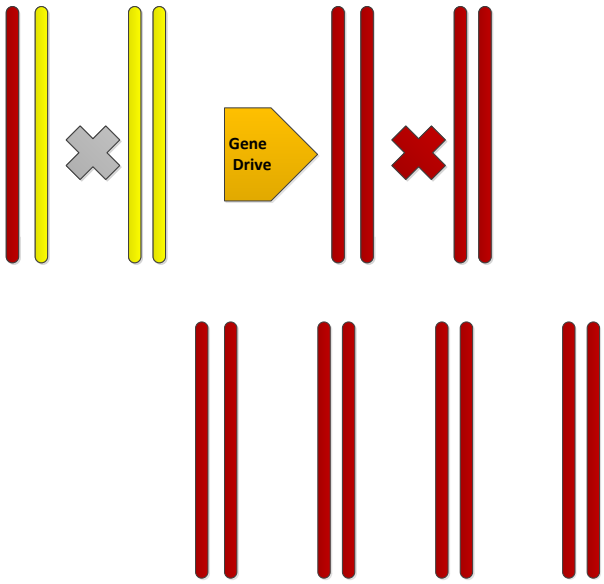
One can imagine the many possible faults in this process. This process, the Cas9 single strand break, the specificity of the Guide RNA all add up to levels of uncertainty of this process.

7.1.1.11 Distribution and Evolution

We now examine the consequences of this approach. First in a Mendelian world as shown below we have four possible offspring. Three of the four express wild type. One will have the mutant gene. If we continue to cross with wild types, then the percent of the mutant will decrease and disappear unless there is some reason for preferentially self-breeding or a natural selection process. Thus in classic Mendelian worlds things are such that the mutant gene will stay suppressed to a reasonable degree.



Now in our Gene Drive world, things are quite different. Remember we have that CRISPR/Cas addition which keeps inserting the mutant gene wherever it can. This we show below.



Thus instead of one in four off springe being mutant now we have any and all off spring being mutant. These mutants mate and their off spring are again all mutant. In no time at all this mutant strain spreads across the species. If the mutant strain is a male only off spring the result is a spreading loss of species until it is completely gone.

That is unless Nature takes some new twist and turn.

7.1.2 Applications

There clearly is a plethora of applications for Gene Drives. We explore some of them here.

7.1.2.1 Disease Control

Many diseases are vector borne. If one could control or eliminate the vector then the disease would disappear. Malaria, Zika, Dengue, and others fall in this group. This most likely will expand to a wide variety of vector borne pathogens.

7.1.2.2 Plant Engineering

Plant bio-engineering has been around now for almost 30 years. This can be a highly productive area for both food stock and horticultural varieties. For example, many invasive species can be controlled while retaining their beneficial effects.

As Barkate and Stephens note:

Different omics platforms have opened the flood gate of potential disease resistance genes that need a more efficient validation pipeline than earlier gene manipulation tools like gene silencing.

Plant–pathogen omics data could be improved even further by reducing the background noise in the biological samples. This can now be achieved, for example, by performing cell-type specific RNA or chromatin profiling with novel tools like INTACT. Cell-type enrichment will help monitor the dynamics of post-translational modifications during plant–pathogen interactions. CRISPR–Cas9 technology has revolutionized gene manipulation capabilities in many species including crops. The multitude of functions that can be performed with CRISPR– Cas9 and its many derivatives make it a molecular tool that will open new opportunities in the complicated world of plant–pathogen interactions and help design durable crop resistance to pathogens.

Only the gene editing function of CRISPR–Cas9 has so far been used in plants and pathogens. However, the future use of dCas9-based tools will also help to unmask the master regulators of disease resistance. GT tools will help integrate omics data in order to fully understand and improve crop defense mechanisms. The complexity of the plant microbiome with good and bad microbes is beginning to be unraveled. CRISPR–Cas9 tools will help future studies of plant–pathogen interactions to transcend individual genes …

7.1.2.3 Human Modification

Imagine if you will that a woman has some genetic disease. Or desires some trait. Then by "gene driving" in the egg it would be possible to modify any of these. Just what human modifications are possible is left to the imagination at this stage.

7.1.3 Observations

We can now make a set of observations regarding Gene Drives.

7.1.3.1 Safety

The National Academy of Sciences NAS report states:

The potential for gene drives to spread throughout a population, to persist in the environment, and to cause irreversible effects on organisms and ecosystems calls for a robust method to assess risks. Environmental assessments and environmental impact statements required by the National Environmental Protection Act, though widely acknowledged as valuable in other contexts, are inappropriate tools to characterize the risks of gene-drive modified organisms. Instead, ecological risk assessment would be beneficial in the context of gene drive research, because this method can be used to estimate the probability of immediate and long-term environmental and public health harms and benefits. Ecological risk assessment allows comparisons among alternative strategies, incorporates the concerns of relevant publics, and can be used to identify sources of uncertainty, making it well-suited to inform research directions and support public policy decisions about emerging gene-drive technologies. Two key features of ecological risk assessments are the ability to trace cause-and-effect pathways and the ability to quantify the probability of specific outcomes.

This approach could also potentially be built into a structured, adaptive process to oversee the release and management of gene-drive modified organisms in the environment. As of May 2016, no ecological risk assessment has yet been conducted for a gene-drive modified organism. Some amount of uncertainty is unavoidable. There is currently sufficient knowledge to begin constructing ecological risk assessments for some potential gene-drive modified organisms, including mosquitoes and mice. In some other cases it may be possible to extrapolate from research and risk analyses focused on other genetically-modified organisms and non-indigenous species. However, laboratory studies and confined field tests (or studies that mimic confined field tests such as large cage trials and greenhouse studies) represent the best approaches to reduce uncertainty in an ecological risk assessment, and are likely to be of greatest use to risk assessors.

This assembly of tools by the genetic engineer has been called "gene drives". In a sense it "drives" certain genes into all members of a species. At least that is the hope. As the Broad Institute states in its licensing statements[42]:

[42] https://www.broadinstitute.org/news/licensing-crispr-agriculture-policy-considerations

Gene drive. This is a way to rapidly spread a new gene throughout an entire species in nature. This approach might be used to block the transmission of malaria by mosquitoes, but has the potential to disrupt ecosystems… After consulting with external experts and careful internal consideration, the Broad Institute has decided to make available non-exclusive research and commercial licenses for the use of CRISPR technology in agriculture -- but with important restrictions. These include: Gene drive: We prohibit the use of the licensed technology for gene drive.

As The Scientist notes[43]:

The United Nations (UN) biodiversity meeting, held in Mexico this month, could have ended poorly for scientists working on gene drives, genetic elements that can perpetuate specific mutations and may help cull dangerous mosquito populations. But in spite of environmental activists pushing the UN to ban gene drives, citing the risk of accidental release, the UN's final agreement—penned December 16—merely urged caution in testing gene drives, Nature reported. Overall, the organization broadly supported further research in synthetic biology. "I'm very relieved," Andrea Crisanti, a molecular parasitologist at Imperial College London who works with gene drives, told Nature. "It would have been a disaster for developing the technology. "By engineering mutations that render organisms infertile or less infectious, then perpetuating these mutations with gene drives, scientists may be able to reduce the occurrence of certain mosquito-borne illnesses and cull invasive species. Gene drives have already been tested in yeast, fruit flies, and mosquitoes, and may soon be enlisted in the fight against malaria. One team hopes to conduct field trials in Africa as soon as 2024.

In a similar fashion Nature states[44]:

When the CBD last met in South Korea in 2014, gene drives were a largely theoretical idea. They are genetic elements that can quickly spread through sexually reproducing populations. In general, an organism's two copies of a gene — known as alleles — each have a 50% chance of being passed on to its offspring. This limits the pace at which a genetic modification can spread through a population. But gene-drive technology tilts the odds, so that a specific change to one allele is inherited by a higher proportion of progeny. In theory, an entire population could quickly carry the same modification. In the past two years, researchers have lab-tested gene drives in yeast, fruit flies and mosquitoes that are based on a gene-editing technology called CRISPR–Cas9. Crisanti's team, for instance, is working on gene drives in the malaria-carrying mosquito Anopheles gambiae that perpetuate mutations causing females to become infertile. Spread of this mutation could mean that mosquito populations plummet to levels that do not support the transmission of malaria. The researchers' project, called Target Malaria, has attracted tens of millions of dollars in funding, and the scientists hope to conduct field trials in Africa as early as 2024. Other groups are developing gene drives to quell island rodents and other pests.

[43] http://www.the-scientist.com/?articles.view/articleNo/47854/title/UN-Rejects-Calls-for-Moratorium-on-Gene-Drive-Research/

[44] http://www.nature.com/news/gene-drive-moratorium-shot-down-at-un-biodiversity-meeting-1.21216

This development takes the next step and it presents a rather double edged sword. It is essential to be watched as we move forward.

7.1.3.2 Accuracy and Repeatability

As we noted previously there are many points of error or failure in these processes. It would be a worthwhile effort if one could develop an error model for this and similar sets of processes. CRISPR errors, insertion errors, DSB repair errors, errors in mitosis, and the list goes on. Not to mention the errors that normally occur to cells. We have also left out such effects as epigenetic methylation effects which can have an overwhelming effect.

7.1.3.3 Inter Species Spreading

One of the concerns is what we term inter-species spread. namely it is possible that the same or different gene transfer may occur from the target species to other species. The risk is low if into a somatic cell perhaps but is significant if into a germ line cell or embryo. Human females for example may have their eggs infiltrated or males the sperm and then result when reproduction occur is infiltration of the new species.

7.1.3.4 Gene Perturbations

The embryo as it progresses may change genes in different generations. The CRISPR may be altered and the result is cutting of the wrong location. Furthermore the replication on the matching chromosome may not work effectively. Double stranded breaks are serious and as we have noted may result in malignancies.

7.1.3.5 Understanding the Dynamics

As Marshall and Hay note:

Gene drive systems are genetic elements capable of spreading into a population even if they confer a fitness cost to their host. We consider a class of drive systems consisting of a chromosomally located, linked cluster of genes, the presence of which renders specific classes of offspring arising from specific parental crosses unviable. Under permissive conditions, a number of these elements are capable of distorting the offspring ratio in their favor. We use a population genetic framework to derive conditions under which these elements spread to fixation in a population or induce a population crash.

Many of these systems can be engineered using combinations of toxin and antidote genes, analogous to Medea, which consists of a maternal toxin and zygotic antidote. The majority of toxin–antidote drive systems require a critical frequency to be exceeded before they spread into a population. Of particular interest, a Z-linked Medea construct with a recessive antidote is expected to induce an all-male population crash for release frequencies above 50%. We suggest molecular tools that may be used to build these systems, and discuss their relevance to the

control of a variety of insect pest species, including mosquito vectors of diseases such as malaria and dengue fever.

The above work was written before the utilization of the CRISPR techniques. The CRISPR approach as one can see is much more aggressive and self-replicating.

7.1.3.6 Weaponizing

One always asks; what is the potential of this technology for harm? Not only accidental harm but deliberate harm, namely weaponized. The answer is a resounding; it has great potential. First the technology is not that sophisticated. Second, the details are in the open literature. Third, the facilities are minimal. Fourth, thousands of graduate students have the capability now and it will wind its way down to the High School level. Fifth, it can easily be spread.

Overall this is just another example of a genie getting out of the jar. We have seen how poorly Governments have deal with Cyber threats, and the Bio threats of this type and others is overwhelming. We all too often focus on Apps and purloined passwords when the implementation of these schemes is all the more important.

7.1.3.7 Ecological Concerns

In banishing a species, deliberately and completely, one makes potentially a massive ecological change. Let us say we eliminate the disease vector mosquitos. o we fully understand any and all unintended consequences. DDT had eliminated much of these vectors then there was the effect of DDT on secondary species and mankind decided to let millions of humans die off to save tens of millions of other species. Are there tradeoffs here as well?

The NAS study does examine the issue of dispersal. They state:

The promise of gene drives is based on the potential spread of the desired gene through an entire area occupied by a species or population. The spread itself occurs via the movement of individuals or gametes from one location to another, with subsequent mating and reproduction. The spread of genes via movement between populations is called gene flow. Understanding the role of gene flow is critical for determining how rapidly a gene drive will spread among populations, whether the goal is to move the drive into additional populations or, conversely to limit its spread.

Understanding gene flow is also vital for estimating the likelihood that the gene drive may move into a non-target population. The diversity of gene flow patterns are influenced by three main factors: the stage of the life cycle in which the movement of individual organisms among populations is most likely, the type of movement through which individuals carry genes among populations, and the spatial scale over which movement typically occurs. Gene flow may occur by the movement of either whole organisms or gametes. For many species, "typical" movement of an individual occurs in specific life cycle stages. For example, in many organisms, movement occurs via dispersal of fertilized eggs, seeds, or spores (as in fungi, ferns, and mosses, for example.).

By contrast, in many animals, movement among populations is most likely when juveniles or young adults of one gender disperse from the area of their birth to establish themselves elsewhere (Graw et al., 2016). In these cases, social interactions can play a critical role in determining individual movement, where an individual settles, and whether movement results in breeding and actual gene flow. The stage of the life cycle in which gene flow occurs can influence the rate at which genes move from one population into another. For example, the passive dispersal of fertilized eggs and seeds can introduce substantial numbers of genes from one population into another, whereas the dispersal of juvenile or adult individuals in search of new habitat will generate much lower rates of gene exchange.

In contrast, many plants and some marine invertebrates disperse primarily through the movement of gametes rather than whole organisms. The most familiar example is wind-borne pollen, which can transport genes across long distances . In many cases, especially when pollen movement is facilitated by insect pollinators, the movement of genes can be quite circumscribed. Gene flow via gametes is fundamentally different from gene flow via movement of individual organisms in two ways. First, it represents sexual transfer of a haploid genome rather than the movement of a diploid genome. Second, it offers a greater possibility of gene flow among closely related species. For example, gamete dispersal can move engineered genes from a target organism into a wild or domesticated relative more quickly and at a higher rate than might occur in hybridization via the movement of seeds among locations (O'Connor et al., 2015). There are four broad types of movement that produce gene flow.

First, individuals move via human assisted dispersal. Human-assisted dispersal is well-recognized as a common avenue for the introduction of unwanted invasive species (Fonzi et al., 2015), but humans also move genotypes from one area to another. This can be accidental, as in the transport of marine organisms in ballast or purposeful, as in the enhancement of game or fishery populations. Human-assisted movement can produce high or low rates of gene flow, depending upon the numbers of individuals transported.

Second, individuals move in response to disruptive events. These can include evacuation in response to wildfires or other sources of rapid habitat destruction or fragmentation. Individuals in aquatic systems can also be transported among locations by flooding events such as flash flooding of streams or sheet flows across large areas…

8 MAB

Antibodies are powerful molecules developed as part of the adaptive immune system. On the one hand, once activated and generated in volume by the B cells, they set out and attach themselves to the antigens on the targeted cells and the Complement system of the Innate immune system takes over and kills the cell. Thus, when we use antigens for vaccines we are essentially priming the Adaptive system to have a large number of Abs ready and able to attack if necessary. On the other hand, recent therapeutics use antibodies to essentially attack certain receptors on cancer cells so that they may subsequently be attacked by a normal immune process. As we had noted in our discussion of the NK cells and of Set Points, there are also Check Points which will stop a normal immune attack, especially on cancer cells. Thus, the approach is to use an Ab to block the blocker, and then allow the immune system to do its job. In this Chapter, we examine how to make specific Abs.

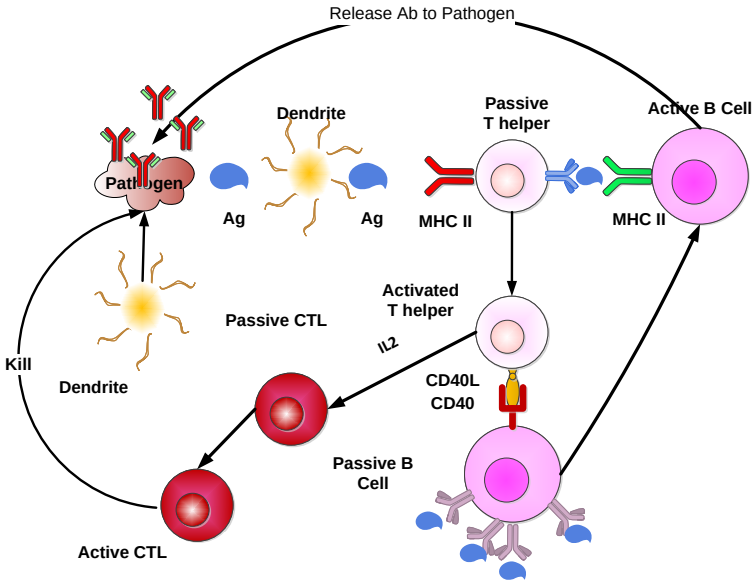
Monoclonal antibodies, Mabs, have been available for decades[45]. Initially they were murine in development but over the past decade we have seen the development of hundreds of new Mabs for a variety of disorders. We will examine them here but we will do so in a manner which constructs another approach to the engineering of immune systems.

We first review some of the elements of the immune system as relates to antibodies and then discuss the Mab development and evolution and finally examine how Mabs have been developed for various disorders.

8.1 Immune System Architecture

Let us return to the overall immune system architecture. The Figure below depicts the complex nature of a Target cell, pathogen, being recognized and attacked. The antibody element is a result of a recognition of some antigen on the Target and the B cells being activated via various mechanisms and then the B cell having a matching Ab being activated to produce those Abs en masse. The result is an explosion of specific Abs and their dissemination throughout the body, their attaching themselves to the Target cells and the activation of the Complement system, the proteins generated in the liver and freely flowing in the blood stream, to neutralize the Target cells.

[45] See Nature Immunology for some historical context. http://www.nature.com/milestones/mileantibodies/Milestones_Poster.pdf and http://www.nature.com/milestones/mileantibodies/collection/index.html Also see Marks, L, The Lock and Key of Medicine: Monoclonal Antibodies and the Transformation of Healthcare, Yale University Press; 1 edition (June 30, 2015).

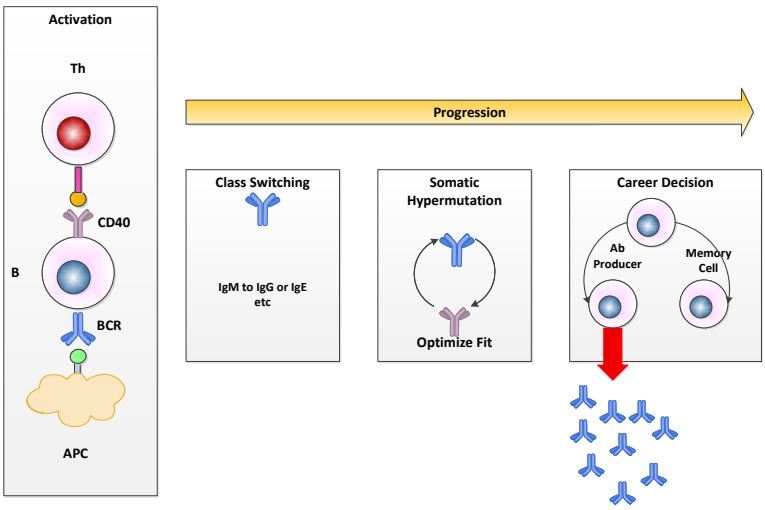


Recall that interaction of antibody with antigen initiates the classical pathway of complement activation. This biochemical cascade of enzymes and protein fragments facilitates destruction of microbes by the membrane attack complex (MAC), by increased opsonization through C3b binding of microbial surfaces and by the production of anaphylotoxins C3a, C5a, and C4a.

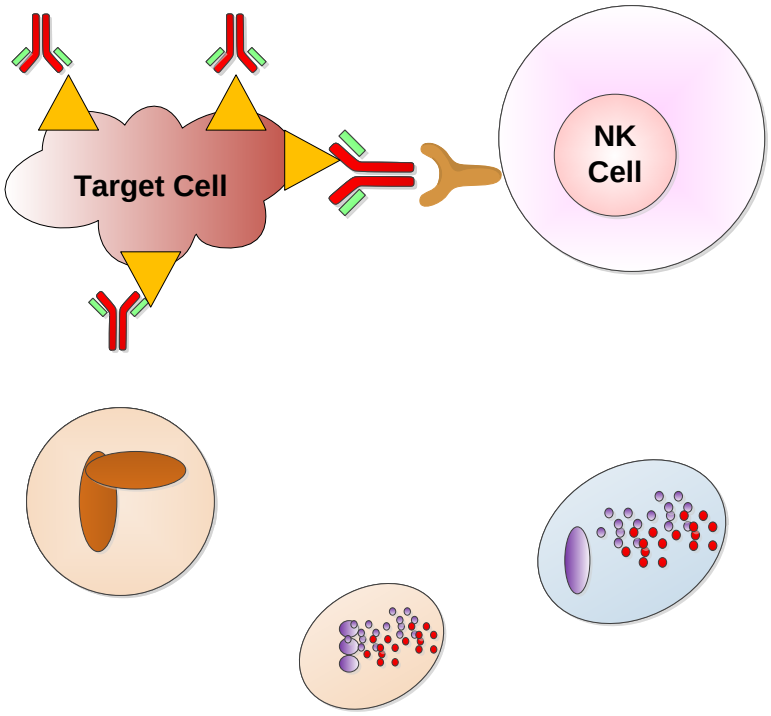
The cascade begins with the activation of component C1. Binding of IgM or IgG antibody to antigen causes a conformational change in the Fc region of the immunoglobulin molecule. This conformational change enables binding of the first component of the classic pathway, C1q. Each head of C1q may bind to a Ch2 domain (within the Fc portion) of an antibody molecule.

Upon binding to antibody, C1q undergoes a conformational change that leads to the sequential binding and activation of the serine proteases C1r and CIs.The C1qrs complex has enzymatic activity for both C4 and C2, indicated by a horizontal bar as either C1qrs or abbreviated as C15. Activation of C1qrs leads to the rapid cleavage and activation of components C4, C2, and C3. In fact, both the classical and mannan-binding lectin (MBL) pathways of complement activation are identical in the cleavage and activation of C4, C2, and C3

The Ab process is detailed more closely below. Note that some activated B cells produce Abs while others are held in abeyance for another future attack.



The above Figure depicts the process of Ab generation. The issue at hand is; what happens with the Ab and what kills off these bad cells? That is particularly important in understanding how to deal with cancer. Cells are eliminated via the interaction of phagocytes as well as the Complement system, part of the innate immune system. As we have noted earlier the NK cells can use the Abs as an indicator of targeting. We show this below along with some of the other phagocytes such as macrophages and neutrophils.



The Complement System is what attacks the Target Cell when it is covered with Abs. As Merle et al note:

Complement is a central part of the innate immunity that serves as a first line of defense against foreign and altered host cells. The complement system is composed of plasma proteins produced mainly by the liver or membrane proteins expressed on cell surface. Complement operates in plasma, in tissues, or within cells. Complement proteins collaborate as a cascade to opsonize pathogens and induce a series of inflammatory responses helping immune cells to fight infection and maintain homeostasis.

The complement system can be initiated depending on the context by three distinct pathways – classical (CP), lectin (LP), and alternative (AP), each leading to a common terminal pathway. In a healthy individual, the AP is permanently active at low levels to survey for presence of pathogens.

Healthy host cells are protected against complement attack and are resistant to persistent low-grade activation. The three pathways are activated on the surface of apoptotic cells, which are constantly generated within the body during normal cellular homeostasis This complement activation is tightly regulated to eliminate dying cells without further activation of other innate or adaptive immune components. Complement is only fully activated in cases of pathogen infection. During an infection, complement leads to inflammation, opsonization, phagocytosis, and destruction of the pathogen and ultimately results in activation of the adaptive immune response. Both inefficient and over stimulation of complement can be detrimental for the host and are associated with increased susceptibility to infections or non-infectious diseases, including autoimmunity, chronic inflammation, thrombotic microangiopathy, graft rejection, and cancer.

The antibody-dependent cell-mediated cytotoxicity can be described as follows. The “tagging" of an invasive organism can attract phagocytic cells and other cytolytic cells. FcRs on NK cells (FcyRIII) and eosinophils (FcyRI, FcbRI, and FcotRI) are IgG-, IgE-, and IgA-specific. The bound cells may be bacteria, protozoa, or even some parasitic worms. As with phagocytic cells, these receptors allow the cytolytic cells to bind invasive organisms “tagged" with IgG, IgE, or IgA antibodies, but rather than engulfment, they use cytolytic mechanisms to kill the “tagged" organisms. This process is termed antibody-dependent cell-mediated cytotoxicity (ADCC). The cytolytic mechanisms used by NK cells and eosinophils in ADCC are similar to some of those used by cytotoxic T cells to kill the intruder.

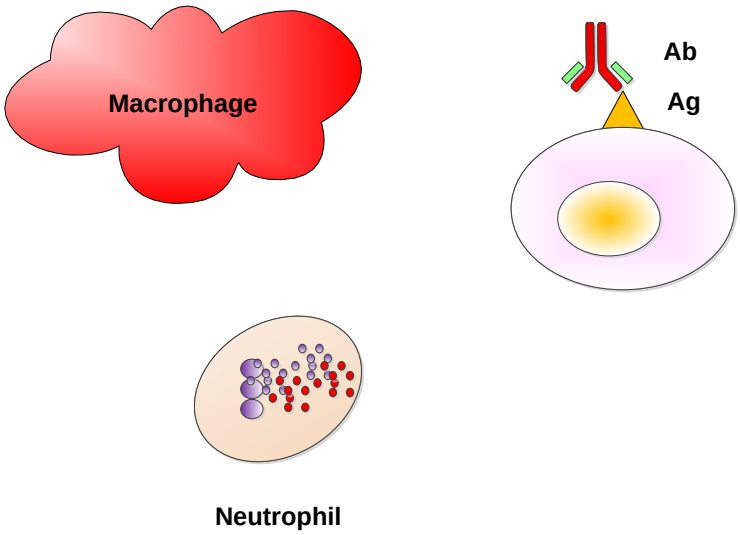
The Complement activation can proceed as follows. The classical pathway of complement is activated by conformational changes that occur in the Fc portion of antibodies upon epitope binding. Antibodies (usually of the IgM and IgG isotypes) facilitate the sequential binding of the C1, C4, C2, and C3 components of the complement system. Like the alternative and mannan-binding lectin pathways, completion of the classical complement pathway results in the production of C3b, a “sticky"

As noted by Merle et al (II):

The main role of complement in pathogen elimination is indirect, namely, the deposition of complement fragments on the surface of pathogen targets, so-called opsonization that allows their recognition, ingestion, and destruction by phagocytic cells, neutrophils,

monocytes, and macrophages. Both IgG antibodies and C3 fragments are the classical opsonins. But complement opsonization, resulting from the direct activation of the AP on pathogens surface allows their elimination by phagocytes before the mounting of a response and the appearance of antibodies.

We demonstrate some of these effects below.



Thus, the process is somewhat simple:

1. Target cells produce an antigen

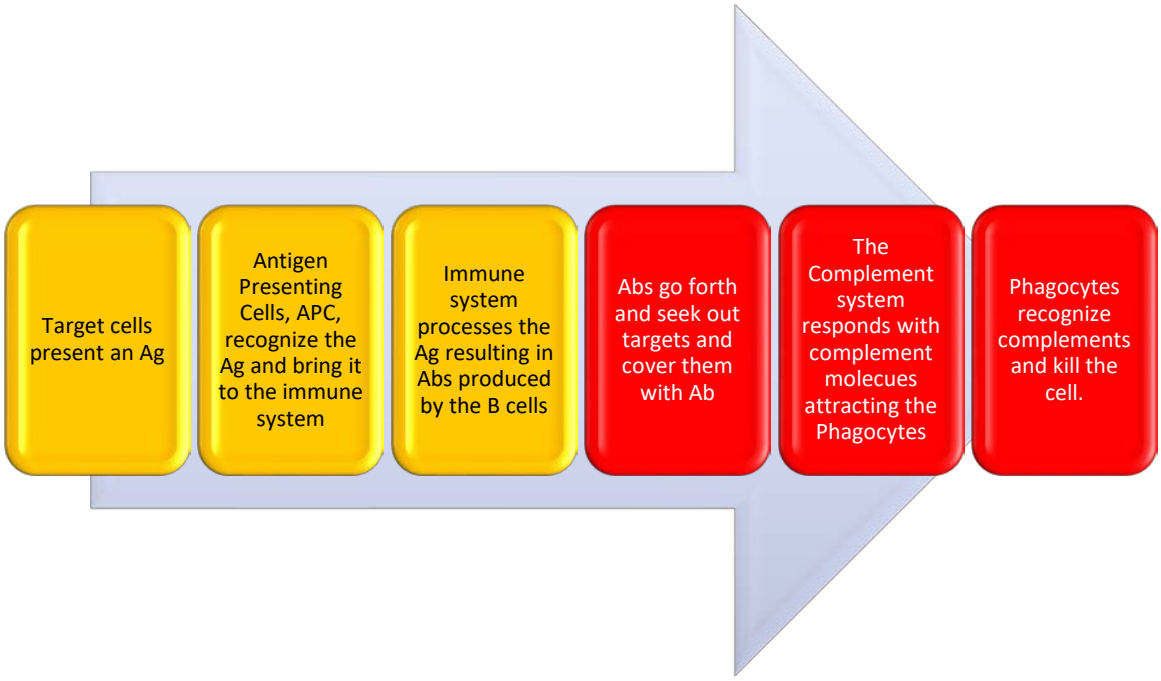
2. Antigen presenting cells see the Ag and carry it to the adaptive system.

3. B cells are activated by the antigen and they produce Abs targeted to the Ag

4. The Abs go out and cover the target cells

5. The Abs attract the Complement system proteins which cover the target as well

6. The phagocytes are brought out to kill off the complement targeted cells.



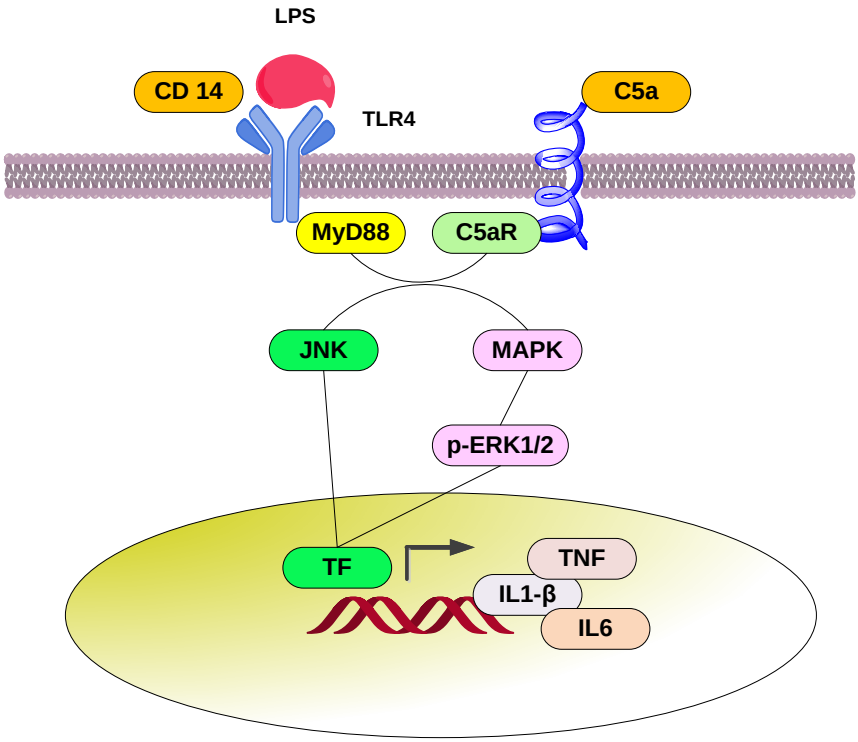
Thus, we see this as an orchestrated process between the elements of the immune system all playing parts in seeking out and destroying invaders. Protection of "self" is a key part of this rather aggressive process and that we leave to the well-established literature.

8.2 The Toll Like Receptor Line

We discussed the Toll Like receptors earlier but they also play a role in Mab action and it is worth a brief discussion. As Merle et al discuss when examine the Complement system they state:

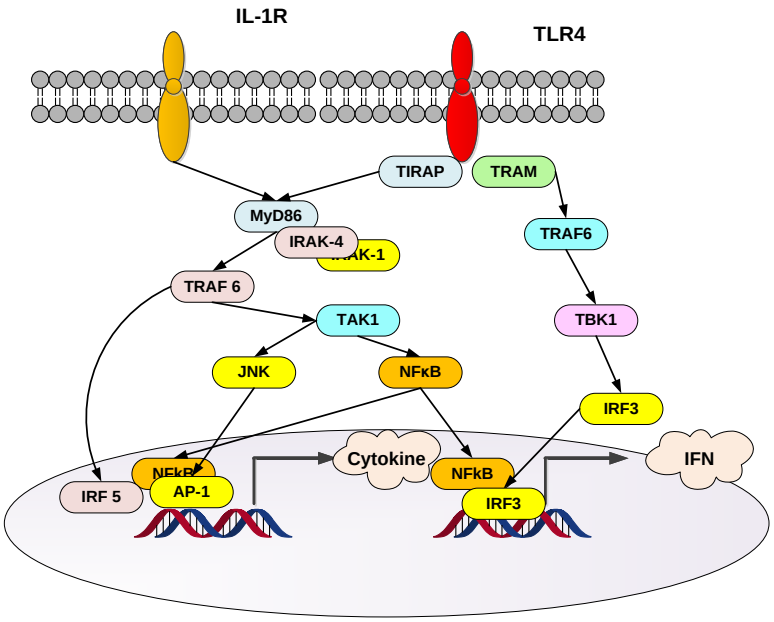
C3a and C5a are able to induce potent inflammatory pathways via their receptors C3aR and C5aR. The implication of intermediates such as NF-kB, MAPK, and c-Jun N-terminal kinase (JNK) in their transduction pathways suggests a potential crosstalk with other pathways, such as those of TLRs. Indeed, complement is involved in TLR-induced inflammation.

They show in the following Figure how this does function:



C5a/C5aR signalingpathwaycancooperatewithTLR-4activationby LPS on macrophages. Intermediate signaling pathways JNK and MAPK are activated and thus lead to proinflammatory effect by TNF-α, IL6, and IL1-β synthesis. On dendritic cells (DCs),TLR-4 and C5aR cooperate in different manner between mice and human. In vivo experiments have demonstrated an implication in Th1cells expansion, whereas in human, an anti-inflammatory role of TLR-4/C5aR collaboration has been described by an antagonized effect on IL-12 and IL-23 synthesis by DC.

Thus, when examining the effects of the complement proteins one must also examine the interactions with other receptors. Further details on this interaction are shown below.



8.3 MAB DEVELOPMENT

Mab development has progressed from mouse models to genetically engineered human analog. It is now possible to accurately design a fully human Ab for use in therapeutic applications. Details are provided in such works as those by Steinitz.

From Steinitz we have:

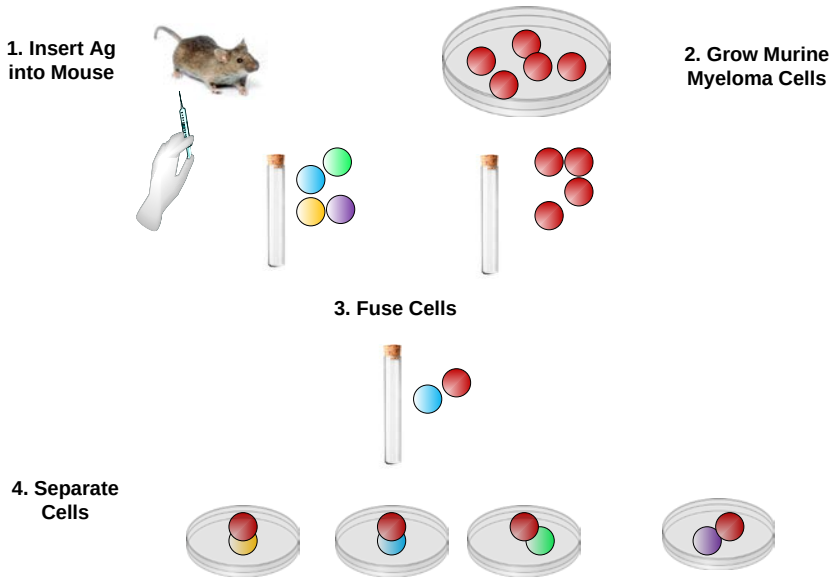
Human antibodies are elicited in response to invading substances (antigens) by B cells. The antigen(s) could be a part of an invading microbe, nonself-cells, or mutated/altered self-cells such as cancer cells.

For a complete immune response various immune cells, in addition to B cells, function together to activate the overall immune system. As a result of the immune response B cells produce antibodies that are specific to an antigen or part (epitope) of an antigen. Antibodies by themselves can destroy or inactivate cells and neutralize substances via a number of mechanisms mediated by nonbinding regions of the antibody.

These mechanisms may require complement and other immune cells, such as NK cells. Because of therapeutic and diagnostic applications of antibodies in human health (control of infectious diseases, autoimmunity, cancer, and other human ailments), they have played a central role in investigative efforts to exploit them to their fullest extent. The first mAbs, of murine origin, were developed more than 35 years ago, as an unlimited source of a single specificity.

However, once in the clinic the xenogeneic nature of the murine mAb resulted in a human anti-murine antibody (HAMA) response in patients that negated the effects of the therapy. Due to these unwanted HAMA responses, various modifications of mAbs to reduce or eliminate the undesired side effects in human were developed which led to the development of chimerized,

humanized, and totally human versions. In addition, innovative in vivo diagnostic and therapeutic applications led to modifications of antibody size [single chain (sFv)] and enhancement of their biological activities



Monoclonal antibodies are created by injecting human cancer cells, or proteins from cancer cells, into mice. The mouse immune systems respond by creating antibodies against these foreign antigens. The murine cells producing the antibodies are then removed and fused with laboratory-grown cells to create hybrid cells called hybridomas. Hybridomas can indefinitely produce large quantities of these pure antibodies. Monoclonal antibodies can be developed to act against cell growth factors, thus blocking cancer cell growth. Monoclonal antibodies can be conjugated or linked to anticancer drugs, radioisotopes, other biologic response modifiers, or other toxins. When the antibodies bind with antigen-bearing cells, they deliver their load of toxin directly to the tumor. Monoclonal antibodies may also be used to preferentially select normal stem cells from bone marrow or blood in preparation for a hematopoietic stem cell transplant in patients with cancer. Monoclonal antibodies achieve their therapeutic effect through multiple direct and indirect mechanisms

1. Can have direct effects in producing apoptosis or programmed cell death.

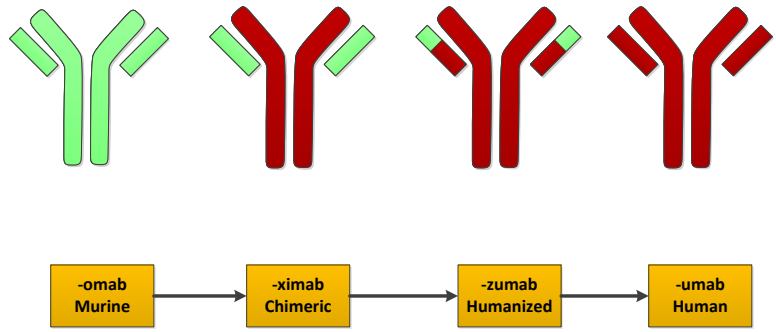
2. Can block growth factor receptors, effectively arresting proliferation of tumor cells.

3. Can bring about anti-idiotype antibody formation in cells that express monoclonal antibodies.

4. Recruiting cells that have cytotoxicity, such as monocytes and macrophages. This type of antibody-mediated cell kill is called antibody-dependent cell mediated cytotoxicity (ADCC),

5. Also bind complement, leading to direct cell toxicity, known as complement dependent cytotoxicity (CDC).

There is an evolution of Mab applications from those which were fully mouse generated which are murine to those fully human. The collection is shown below. Namely we have a murine, chimeric, humanized and human. Recall that the binding to the Ab occurs at the epitope site on one of the two arms.



In the current therapeutic market, most if not all are human genetically engineered and referred to as -umab.

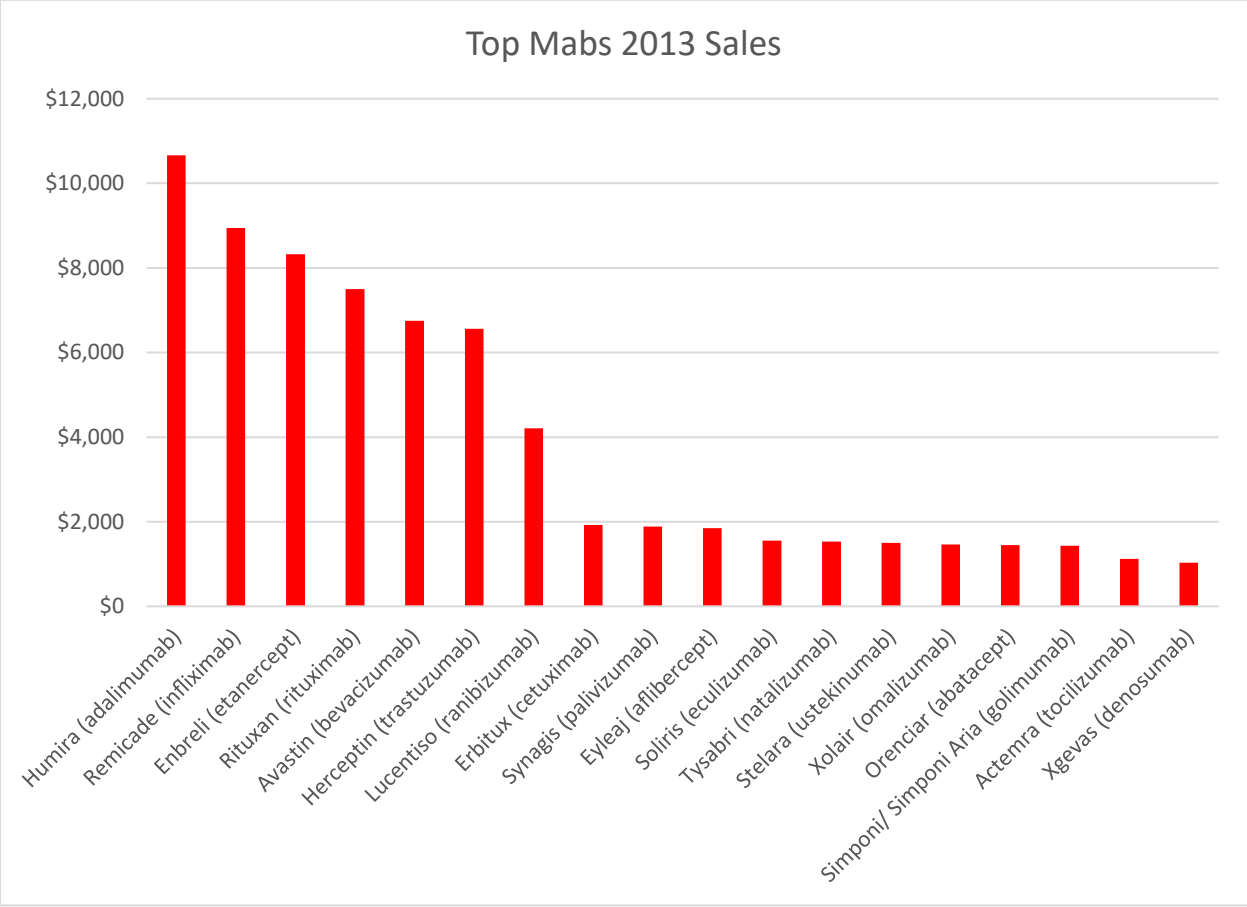
8.4 MAB APPLICATION

The following Table is, as modified, from Galluzzi et al and depicts many of the current Mabs and their applications.:

Alemtuzumab	Chronic lymphocytic leukemia	2001	Selective recognition/opsonization of $CD52_+$ neoplastic cells
Bevacizumab	Colorectal carcinoma Glioblastoma multiforme Cervical carcinoma Lung carcinoma Renal cell carcinoma	2004	VEGFA neutralization
Brentuximab vedotin	Anaplastic large cell lymphoma Hodgkin's lymphoma	2011	Selective delivery of MMAE to $CD30_+$ neoplastic cells
Blinatumumab	Acute lymphoblastic leukemia	2014	CD3- and CD19-specific BiTE
Catumaxomab	Malignant ascites in patients with $EPCAM_+$ cancer	2009	CD3- and EPCAM-specific BiTE
Cetuximab	Head and neck cancer Colorectal carcinoma	2004	Inhibition of EGFR signaling
Denosumab	Breast carcinoma Prostate carcinoma Bone giant cell tumors	2011	Inhibition of RANKL signaling
Gemtuzumab ozogamicin	Acute myeloid leukemia	2000	Selective delivery of calicheamicin to $CD33_+$ neoplastic cells
Ibritumomab tiuxetan	Non-Hodgkin lymphoma	2002	Selective delivery of ^{90}Y or ^{111}In to $CD20_+$ neoplastic cells
Panitumumab	Colorectal carcinoma	2006	Inhibition of EGFR signaling
Pertuzumab	Breast carcinoma	2012	Inhibition of HER2 signaling
Obinutuzumab	Chronic lymphocytic leukemia	2013	Selective recognition/opsonization of $CD20_+$ neoplastic cells
Ofatumumab	Chronic lymphocytic leukemia	2009	Selective recognition/opsonization of $CD20_+$ neoplastic cells
Ramucirumab	Gastric or gastroesophageal junction adenocarcinoma	2014	Inhibition of KDR signaling
Rituximab	Chronic lymphocytic leukemia Non-Hodgkin lymphoma	1997	Selective recognition/opsonization of $CD20_+$ neoplastic cells

Siltuximab	Multicentric Castleman's disease	2014	IL-6 neutralization
Tositumomab	Non-Hodgkin lymphoma	2003	Selective recognition/opsonization of, or selective delivery of ^{90}Y or ^{111}In to, $CD20^{+}$ neoplastic cells
Trastuzumab	Breast carcinoma Gastric or gastroesophageal junction adenocarcinoma	1998	Selective recognition/opsonization of, or selective delivery of mertansine to, $HER2^{+}$ cancer cells
Lenalidomide	Mantle cell lymphoma Myelodysplastic syndrome Multiple myeloma	2005	IKZF degradation and immunomodulation
Pomalidomide	Multiple myeloma	2013	IKZF degradation and immunomodulation
Thalidomide	Multiple myeloma	2006	IKZF degradation and immunomodulation
Trabectedin	Soft tissue sarcoma Ovarian carcinoma	2007	Reprogramming of tumor- associated macrophages

From the work of Ecker et al we have the following Table which complements the above:



8.5 Issues with Ab Approaches

There are a number of obstacles to successful therapy with monoclonal antibodies:

1. Antigen distribution of malignant cells is highly heterogeneous, so some cells may express tumor antigens, while others do not.

2. Antigen density can vary as well, with antigens expressed in concentrations too low for monoclonal antibodies to be effective.

3. Tumor blood flow is not always optimal. If monoclonal antibodies need to be delivered via the blood, it may be difficult to reliably get the therapy to the site.

4. High interstitial pressure within the tumor can prevent the passive monoclonal antibodies from binding.

5. Since monoclonal antibodies are derived from mouse cell lines, the possibility of an immune response to the antibodies exists. This response not only decreases the efficacy of monoclonal antibody therapy, but also eliminates the possibility of re-treatment.

6. Very rarely do we see cross-reactivity with normal tissue antigens—in general target antigens that are not cross reactive with normal tissue antigens are chosen. Despite these obstacles, there has been tremendous success in the clinical application of monoclonal antibodies in hematologic malignancies and solid tumors.

It should also be noted that Abs when sent out by the immune system basically attach to cells with Ag and then attract the Complement system to attack and destroy. However, when used to be a Checkpoint Inhibitor such as in PD-1 blockade, the attach to PD-1 yet do not activate the Complement system, they allow the immune system to attack in a different manner. Perhaps this is a difference in functioning or perhaps not. It has been noticed in many Mab trials that there are secondary effects, which frankly would be expected.

Baldo presents the following list of Mabs when he discusses their potential adverse responses.

Generic Name	Type of Mab	Target	Action	Approved Fo	Trade Name
Catumaxomab	rat IgG2b / Mouse IgG2a bispecfic	epCaMc/CD3d	Binds both epCaM on tumor cell and CD3 on T cell	Malignant ascites	removab®
Ibritumomab tiuxetane	Murine IgG1κ	CD20	Binds B cells and kills with aDCC,f CDCf and radiatione	Non-Hodgkin lymphoma	Zevalin®
Tositumomab-131I	Murine IgG2aλ	CD20	Binds to and kills B cells with 131I	Non-Hodgkin lymphoma	Bexxar®
-ximabs					
Brentuximab vedoting	Chimeric IgG1κ	CD30h	antimitotic MMaeg	anaplastic large cell lymphoma; Hodgkin lymphoma	adcetris®

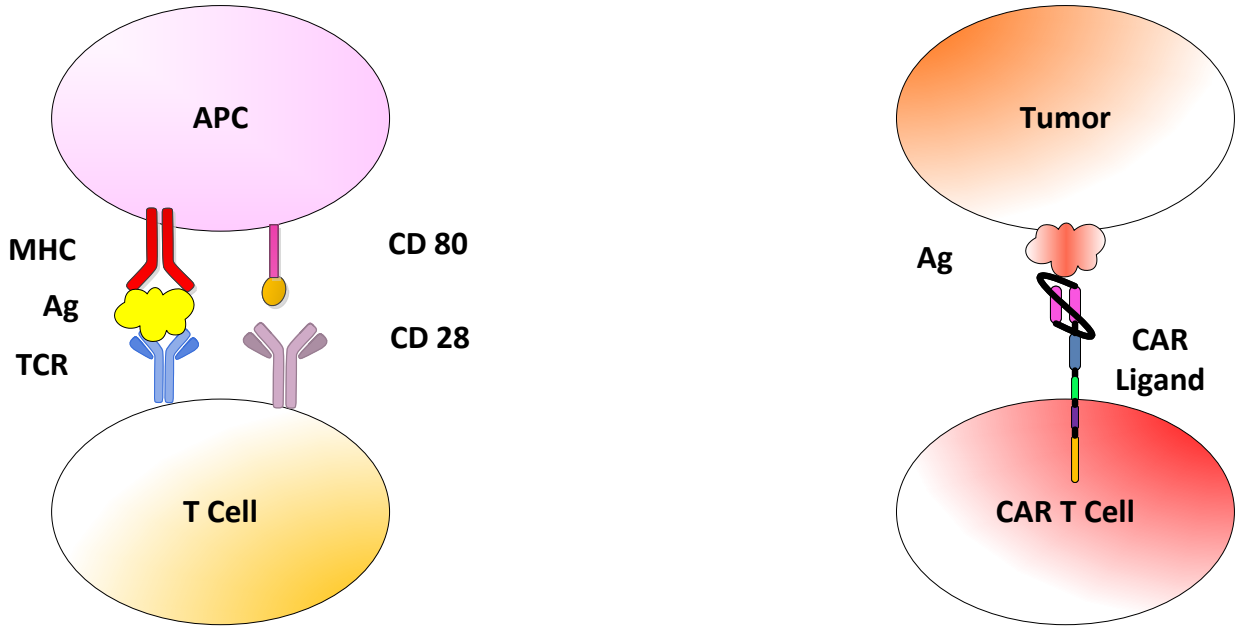
Cetuximab	Chimeric IgG1κ	eGFri	Binds to eGFr and turns off cell divisionj	Colorectal cancer; head and neck cancers	erbitux®
rituximab	Chimeric IgG1κ	CD20	Binds to CD20 on B cells leading to cell death	Non-Hodgkin lymphoma	MabThera® rituxan®
-zumabs					
alemtuzumab	Humanized IgG1κ	CD52k	eliminates lymphocytes	Chronic lymphocytic leukemia	Campath-1H®
Bevacizumab	Humanized IgG1κ	veGFl	angiogenesis inhibitor	Colorectal, lung, kidney, brain cancers	avastin®
Pertuzumab	Humanized IgG1κ	Her2m	Inhibits dimerization of Her2 with other Her receptors	Metastatic breast cancer	Perjeta®
Trastuzumab	Humanized IgG1κ	Her2	Prevents overexpression of Her2	Breast cancer	Herceptin®
Trastuzumab emtansinen	Humanized IgG1κ	Her2	mab-drug conjugate. as for trastuzumab plus cytotoxic effect of mertansine (DM1)o	advanced metastatic breast cancer	Kadcyla™
-umabs					
Denosumab	Human IgG2κ	raNKLp	Inhibits activation of osteoclasts by raNKL	Bone metastases; Giant cell tumor of the bone (GCTB)	Prolia® Xgeva®
Ipilimumab	Human IgG1κ	CTLa-4q	Blocks interaction of CTLa-4 with its ligandsr and enhances T cell activation	Metastatic melanoma	yervoy®
Ofatumumab	Human IgG1κ	CD20	Binds to CD20 on B cell causing cell death	Chronic lymphocytic leukemia	arzerra®
Panitumumab	Human IgG2κ	eGFri	Binds to and prevents activation of eGFr	Colorectal cancer	vectibix®

9 CAR T

The immune system is a powerful tool that can be used in a variety of ways. One problem is that it seems that every day we discover another subtlety regarding how this functions. It is a tool, and a very powerful tool, that can be used as a scalpel or a butchering ax. With the advent of CARs, specifically designed killer T cells, CTLs or cytotoxic T cells, one can attack cancer cells, however at times this tool can explode in our hands. This paper is an attempt to examine the CAR T Cells as a tool which can be engineered. The problem we face however is that in engineering the tool we oftentimes do not have a full grasp of its effects.

Our intent herein is not to provide a detailed up to date review of CARs but to provide a summary introduction to the potential they provide. This area is still very much a work in progress and as such is subject to ongoing change.

CAR T cells combine the antigen recognition domain of an antibody with the intracellular signalling domains into a single cell surface receptor. The CAR T cells allows for the MHC independent antigen recognition and secondly enables the T cells to execute its cytotoxic behavior. A CART T cell is a genetically modified T cell wherein the modified cell can attack a broad set of specific targets and do so even though the target is not fully expressing what would normally be required. The CAR T model below is compared to the CTL model discussed earlier. Note by creating the CAR ligand we can identify and attach and activate when seeing the antigen only. The MHC requirement is removed and the only issue is engineering this CAR molecule and then placing it in the patient's T cells, growing them, and returning them to the patient. Then, in essence we have designed a target specific T cell attack (see Lee at al).



9.1 History and Principles

Steven Rosenberg has been studying how best to use the immune system to fight cancer. His 1992 was a prescient piece that laid out the future opportunities. From then until now, some 25 years, we know a great deal about the immune system which was lacking then and furthermore we have a wealth of tools to manipulate the cells involved.

From Kahilil et al we have an introduction to CARs which provide continuity from the work on monoclonal antibodies, MABs:

In the past decade, advances in the use of monoclonal antibodies (mAbs) and adoptive cellular therapy to treat cancer by modulating the immune response have led to unprecedented responses in patients with advanced-stage tumors that would otherwise have been fatal. To date, three immune-checkpoint-blocking mAbs have been approved in the USA for the treatment of patients with several types of cancer, and more patients will benefit from immunomodulatory mAb therapy in the months and years ahead.

Concurrently, the adoptive transfer of genetically modified lymphocytes to treat patients with hematological malignancies has yielded dramatic results, and we anticipate that this approach will rapidly become the standard of care for an increasing number of patients. In this Review, we highlight the latest advances in immunotherapy and discuss the role that it will have in the future of cancer treatment, including settings for which testing combination strategies and 'armored' CAR T cells are recommended.

From Batlevi et al we have a discussion on the flow from MABs to CARs with a nexus to checkpoint inhibitors, namely PD-1 inhibitors:

The success of the anti-CD20 monoclonal antibody rituximab in the treatment of lymphoid malignancies provided proof-of-principle for exploiting the immune system therapeutically. Since the FDA approval of rituximab in 1997, several novel strategies that harness the ability of T cells to target cancer cells have emerged.

Reflecting on the promising clinical efficacy of these novel immunotherapy approaches, the FDA has recently granted 'breakthrough' designation to three novel treatments with distinct mechanisms.

First, chimeric antigen receptor (CAR)-T-cell therapy is promising for the treatment of adult and pediatric relapsed and/or refractory acute lymphoblastic leukemia (ALL).

Second, blinatumomab, a bispecific T-cell engager (BiTE®) antibody, is now approved for the treatment of adults with Philadelphia-chromosome-negative relapsed and/or refractory B-precursor ALL.

Finally, the monoclonal antibody nivolumab, which targets the PD-1 immune-checkpoint receptor with high affinity, is used for the treatment of Hodgkin lymphoma following treatment failure with autologous-stem-cell transplantation and brentuximab vedotin.

Herein, we review the background and development of these three distinct immunotherapy platforms, address the scientific advances in understanding the mechanism of action of each therapy, and assess the current clinical knowledge of their efficacy and safety. We also discuss future strategies to improve these immunotherapies through enhanced engineering, biomarker selection, and mechanism-based combination regimens.

One of the observations when dealing with cancer and the immune system is that once when on tries a specific approach one often finds new mechanisms which can either be used or must be thwarted.

From Jackson et al there is a discussion of the work of CARs using CD-19 targets:

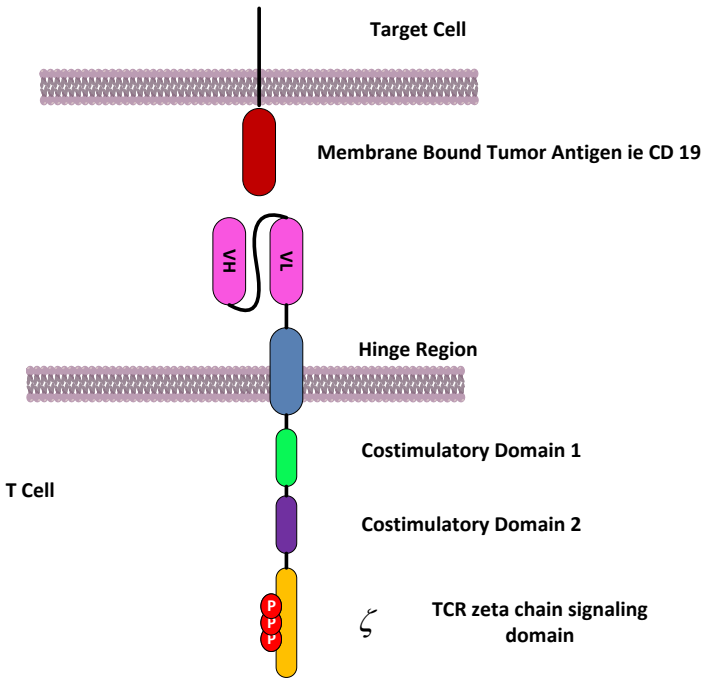
The engineered expression of chimeric antigen receptors (CARs) on the surface of T cells enables the redirection of T-cell specificity. Early clinical trials using CAR T cells for the treatment of patients with cancer showed modest results, but the impressive outcomes of several trials of CD19-targeted CAR T cells in the treatment of patients with B-cell malignancies have generated an increased enthusiasm for this approach. Important lessons have been derived from clinical trials of CD19-specific CAR T cells, and ongoing clinical trials are testing CAR designs directed at novel targets involved in hematological and solid malignancies.

In this Review, we discuss these trials and present strategies that can increase the antitumor efficacy and safety of CAR T-cell therapy. Given the fast-moving nature of this field, we only discuss studies with direct translational application currently or soon-to-be tested in the clinical setting.

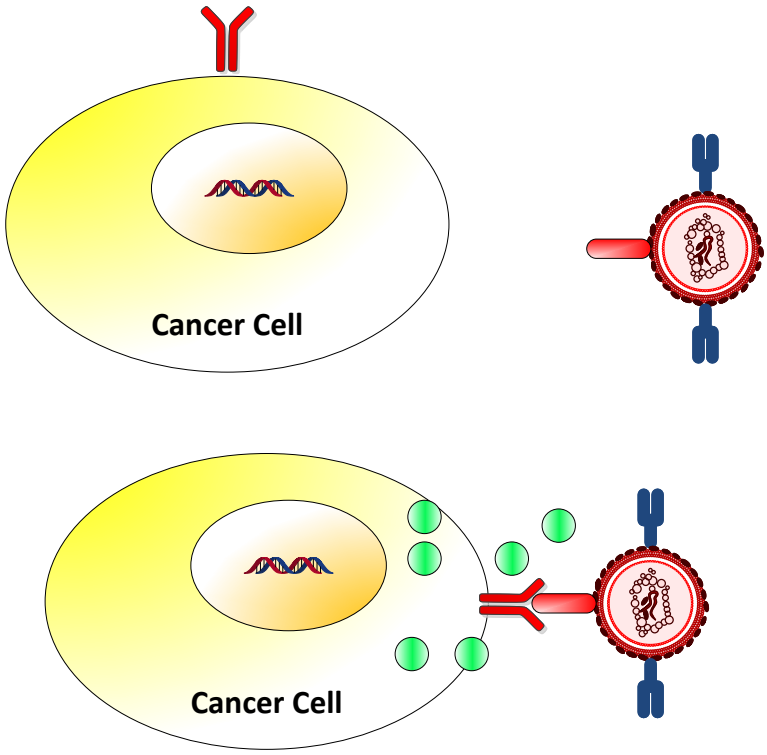
9.1.1 CAR T Cells

CAR T cells are chimeric antigen receptors on T cells. Chimeric because one designs them specifically for the target cells and essentially crated a multiheaded receptor that matches the antigen presented by the tumor cell.

We provide a simple example below for a third-generation CAR:



The function of this designed T cell it to allow a normal CTL, killer T cell, attach to a cancer cell with a recognizable antigen, and then to do what CTLs do well, allow the attacked cell to go into apoptosis, and just disappear, its constituents being used elsewhere.



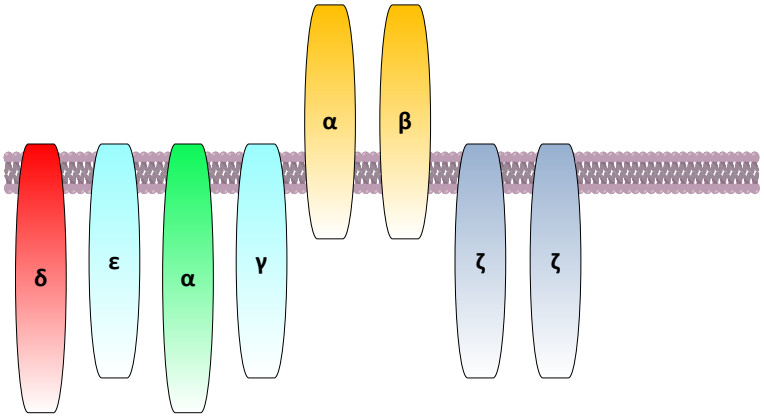
We demonstrate this process graphically above.

9.1.2 T Cells

We review some of the key functions of T cells. The two types of T cells of interest are T helper and T killer or cytotoxic T cells, CTL. The CTL is the prime target of interest for it is the cell which can attach to a tumor cell and effect apoptosis of the tumor cell by its normal operations. The T helper supports the CTL by expressing IL-2 which allows for proliferation of that specific CTL type.

9.1.3 T Cell Dynamics

The CTL has surface receptors as shown below. Two are extending well beyond the cell wall and the remaining four are below the cell wall and provide for intra cellular activation. The complex acts in unison attaching to targeted cells. Now the essence of CARs is to modify this receptor so as to effect targeting of tumor cells and their exposed antigens.

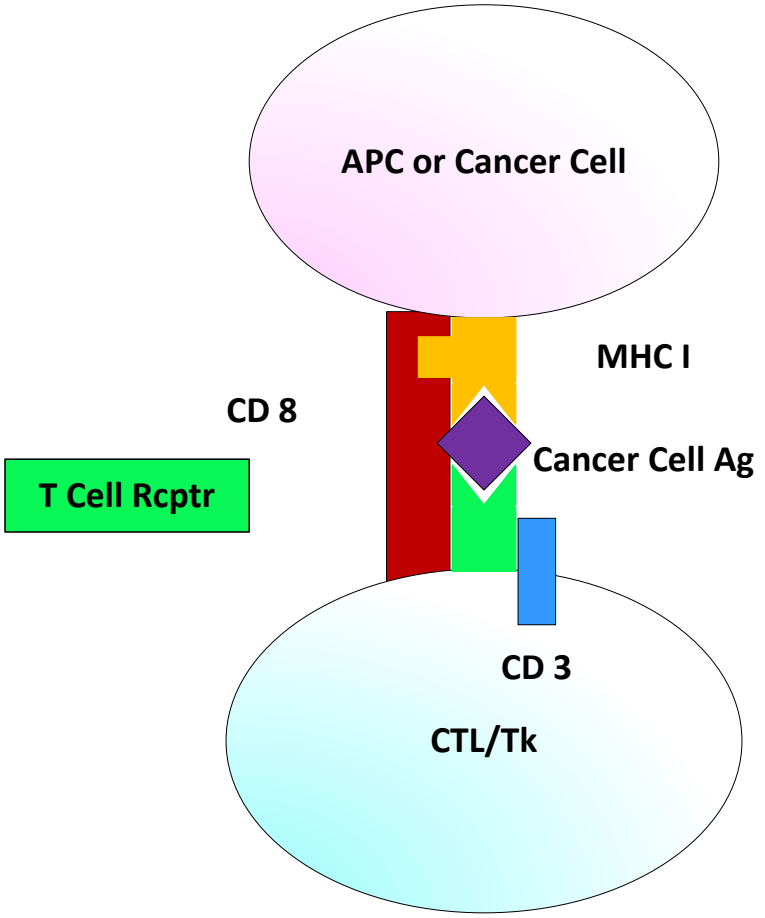


This CTL binding process is shown below. Simply the process is as follows:

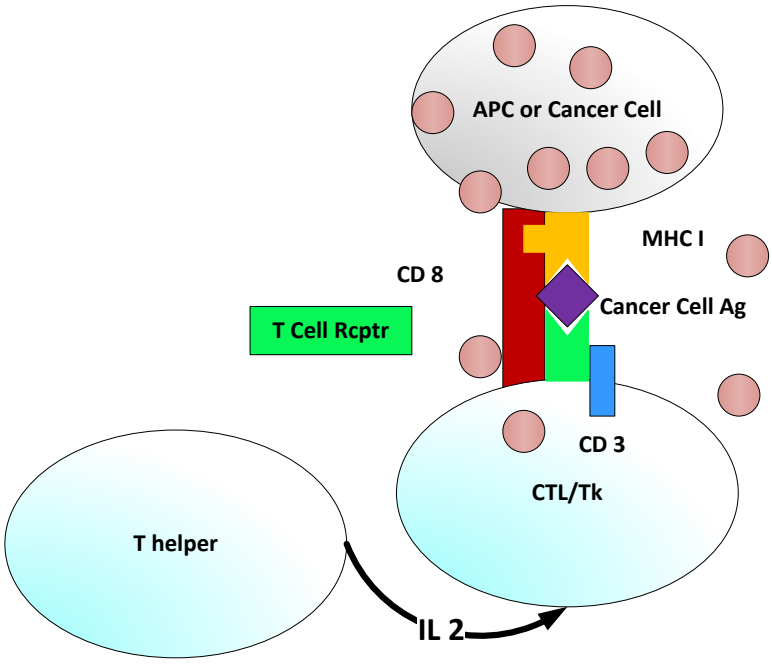
1. An antigen presenting cell, APC, in this case a tumor cell, presents an antigen using the MHC I molecule. Also, the tumor cell may have another surface protein that results in the presentation of a tumor specific surface molecule like CD-19 in the case of hematological malignancies. The process starts with the ability to identify this molecule.

2. Then the CTL has a matching or cognate receptor which aligns with the MHC I and Ag combination and it attaches itself, and via CD-8 strongly binds to the cell, also using CD-3.

3. Upon binding the CTL can release cytokines or equivalents that result in the apoptosis of the cell.



We show the apoptosis below. Here the bound CTL recognizes the cancer cell and then releases apoptosis inciting proteins.



Thus, for any cancer cell we should be able to use this process, if we first know the Ag that is presented and second if we can create a receptor on a T killer cell, CTL, that recognizes that ligand and in turn can activate the apoptotic process.

In the simplest terms this is how we might proceed.

1. Extract a tumor cell.

2. Ascertain the surface molecules and determine which one is unique to that type of cell and NOT common in other cells. You don't want the CTLs attacking everything.

3. Create a binding receptor for that ligand.

4. Extract the patient's CTL and insert by some reverse transcription manner, or CRISPR type approach the genes for that designed receptor.

5. Grow these modified cells in vitro using IL-2 or the like.

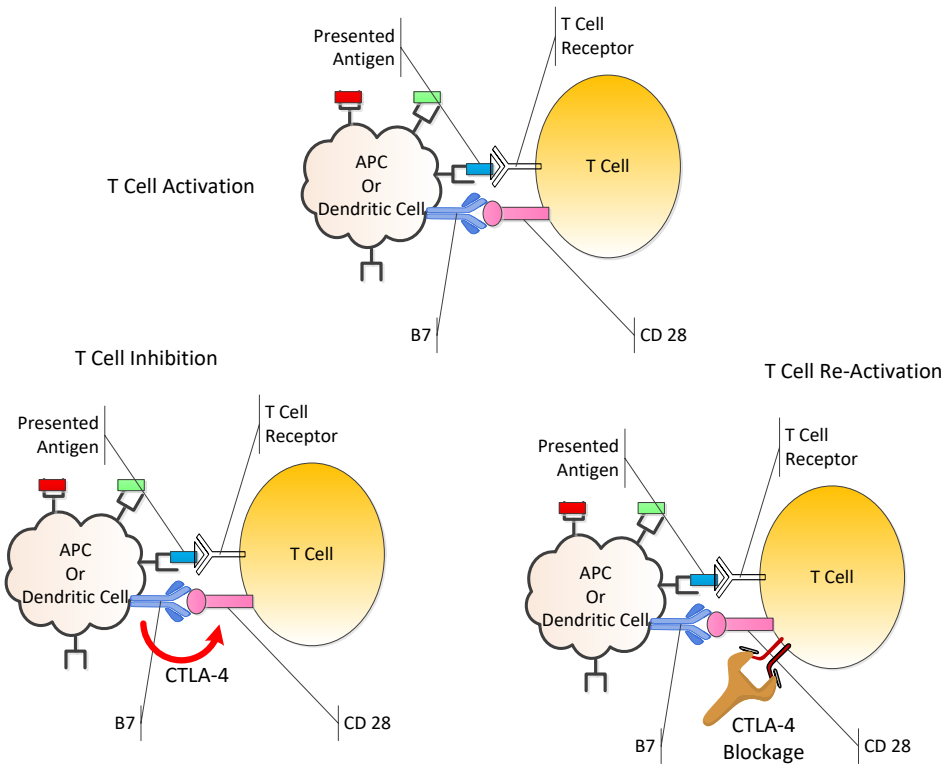
6. Insert these back in the patient.

This is the "back of the envelope" approach to CAR therapy. Of course, there are many obstacles and the approach uses tools which may have to be gathered from afar. But as those who have developed CARs have shown it is doable.

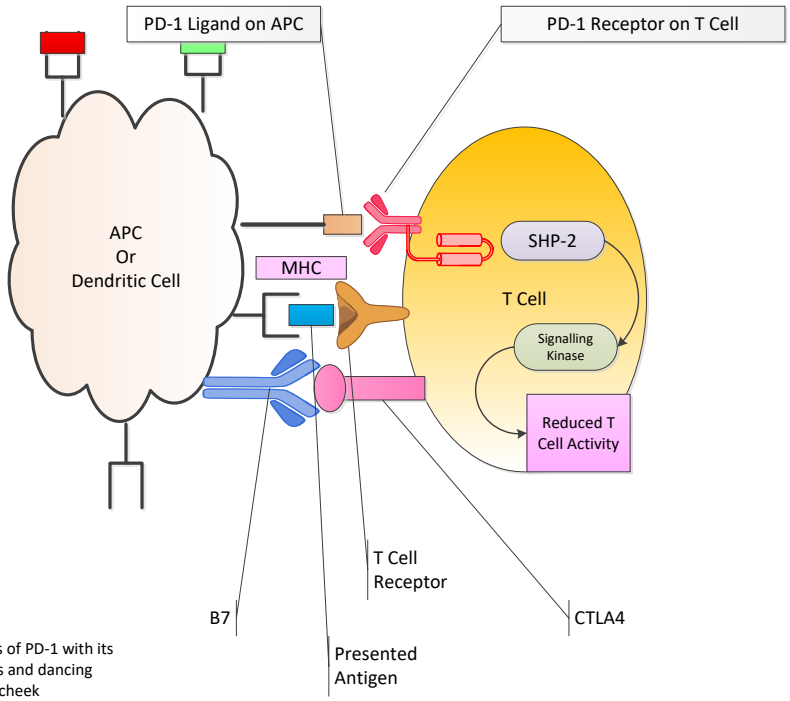
9.1.4 PD-1 Pathways

Now what we have described above is not that simple. There are what are called a variety of "Checkpoint Inhibitors" that are an integral part of the control mechanisms of the immune system so that it does not go wild and destroy itself.

Let us begin with a brief review of PD-1 pathways. We have previously discussed the CTLA-4 blockage and the current approaches used to inactivate that element of T cell suppression. We summarize that again in the figure below.



Now CTLA-4 is not the only inhibitor of T cell action. PD-1 also can be activated and thus suppress T cell activity. This means that is we can find a way to inactivate or inhibit PD-1 then we have another way to seek possible activation of the T cells. In fact, perhaps we can do both and secure a super active T cell base. That is in essence the Wolchok approach. We depict this in the figure below.



Freeman, Structures of PD-1 with its ligands: Sideways and dancing cheek to cheek

The paper by Okazaki and Honjo in 2007 also details many of the critical elements regarding the PD-1 and its ligands. It details many of the recognized disease states as well. As they state:

Since the discovery of PD-1 in 1992, the biological function of PD-1 remained mystery for many years. Generation of Pdcd1mice and the discovery of its ligands turned around the situation and the function of PD-1 was unveiled thick and fast in these 5 years. Consequently, it became clear that PD-1 plays critical roles in the regulation of autoimmunity, tumor immunity, infectious immunity, transplantation immunity, allergy and immune privilege. The development of autoimmune diseases by Pdcd1 mice especially enchanted clinicians and promoted clinical research as well.

Currently, many groups are trying to generate not only PD-1 antagonists for the treatment of cancer and infectious diseases but also PD-1 agonists for the treatment of autoimmune diseases, allergy and transplant rejection. Among these, humanized antibody against human PD-1 was approved by Food and Drug Administration of the United States as an investigational new drug in August 1, 2006. Clinical trials will test its clinical efficacy on cancer and infectious diseases.

Now we can examine the features of PD-1. As Freeman states:

T cell activation requires a TCR mediated signal, but the strength, course, and duration are directed by costimulatory molecules and cytokines from the antigen-presenting cell (APC). An unexpected finding was that some molecular pairs attenuate the strength of the TCR signal, a process termed co-inhibition.

The threshold for the initiation of an immune response is set very high, with a requirement for both antigen recognition and costimulatory signals from innate immune recognition of ‘‘danger’’ signals. Paradoxically, T cell activation also induces expression of co-inhibitory receptors such as programmed death-1 (PD-1).

Cytokines produced after T cell activation such as INF- and IL-4 up-regulate PD-1 ligands, establishing a feedback loop that attenuates immune responses and limits the extent of immune-mediated tissue damage unless overridden by strong costimulatory signals. PD-1 is a CD28 family member expressed on activated T cells, B cells, and myeloid cells. In proximity to the TCR signaling complex, PD-1 delivers a co-inhibitory signal upon binding to either of its two ligands, PD-L1 or PD-L2.

Engagement of ligand results in tyrosine phosphorylation of the PD-1 cytoplasmic domain and recruitment of phosphatases, particularly SHP2

Additional insight can also be provided by examining the regulatory T cells as well. As Francisco et al state:

Regulatory T cells (Tregs) and the PD-1: PD-ligand (PD-L) pathway is both critical to terminating immune responses. Elimination of either can result in the breakdown of tolerance and the development of autoimmunity. The PD-1: PD-L pathway can thwart self-reactive T cells

and protect against autoimmunity in many ways. In this review, we highlight how PD-1 and its ligands defend against potentially pathogenic self-reactive effector T cells by simultaneously harnessing two mechanisms of peripheral tolerance: (i) the promotion of Treg development and function and (ii) the direct inhibition of potentially pathogenic self-reactive T cells that have escaped into the periphery.

Treg cells induced by the PD-1 pathway may also assist in maintaining immune homeostasis, keeping the threshold for T-cell activation high enough to safeguard against autoimmunity. PD-L1 expression on non-hematopoietic cells as well as hematopoietic cells endows PD-L1 with the capacity to promote Treg development and enhance Treg function in lymphoid organs and tissues that are targets of autoimmune attack. At sites where transforming growth factor-β is present (e.g. sites of immune privilege or inflammation), PD-L1 may promote the de novo generation of Tregs.

9.1.5 CAR Cells

CAR cells are essentially engineered T cells, specifically cytotoxic T lymphocytes, CTL, engineered to target specific cells such as those in various hematopoietic cell lines. such as leukemias and lymphomas. There is no fundamental reason that they cannot be used for solid tumors but there are certain operational barriers which must be overcome.

As Kershaw et al note:

There are two main types of antigen receptors used in genetic redirection.

The first utilizes the native alpha and beta chains of a TCR specific for tumor antigen.

The second is termed a chimeric antigen receptor (CAR), which is composed of an extracellular domain derived from tumor-specific antibody, linked to an intracellular signaling domain. Genes encoding these receptors are inserted into patient's T cells using viral vectors to generate tumor reactive T cells….

The specificity of CARs is derived from tumor-specific antibodies, which are relatively simple to generate through immunization of mice. Recombinant techniques can be used to humanize antibodies, or mice expressing human immunoglobulin genes can be used to generate fully human antibodies. Single-chain variable fragments of antibodies are used in the extracellular domain of CARs, which are joined through hinge and transmembrane regions to intracellular signaling domains.

As Miller and Sadelain note:

The advent of gene transfer technologies, in particular those enabling the transduction of human T lymphocytes using gibbon ape leukemia virus envelope-pseudotyped g-retroviral vectors, created new opportunities for immune intervention based on T cell engineering. Patients' T cells, easily accessible in peripheral blood, can be genetically instructed to target tumors by

transduction of receptors for antigen, utilizing either the physiological TCR or synthetic receptors now known as CARs.

Both approaches have shown clinical successes, particularly in melanoma, targeting NYESO1, and in acute lymphoblastic leukemia, CARs are artificial, composite receptors for antigen that integrate principles of B cell and T cell antigen recognition. They are particularly attractive in that they elude human leucocyte antigen (HLA) restriction and are thus applicable to all patients irrespective of their HLA haplotypes, unlike TCRs. CARs may also overcome HLA downregulation by tumors, which deprives T cells of a ligand for their endogenous TCR.

The critical function of CARs is, however, not to merely target the T cells to a tumor antigen, but to enhance T cell function. Thus, effective CARs further integrate principles of T cell costimulation and provide a broad spectrum of functional enhancements acquired by directly soliciting selected costimulatory pathways

Juillerat et al note:

Adoptive immunotherapy using engineered T-cells has emerged as a powerful approach to treat cancer. The potential of this approach relies on the ability to redirect the specificity of T cells through genetic engineering and transfer of chimeric antigen receptors (CARs) or engineered TCRs1. Numerous clinical studies have demonstrated the potential of adoptive transfer of CAR T cells for cancer therapy but also raised the risks associated with the cytokine-release syndrome (CRS) and the "on-target off-tumor" effect.

To date, few strategies have been developed to pharmacologically control CAR engineered T-cells and may rely on suicide mechanisms. Such suicide strategies leading to a complete eradication of the engineered T-cells will result in the premature end of the treatment. Consequently, implementing non-lethal control of engineered CAR T-cells represents an important advancement to improve the CAR T-cell technology and its safety.

Small molecule based approaches that rely on dimerizing partner proteins have already been used to study, inter alia, the mechanism of T-cell receptor triggering15. Very recently, Lim and colleagues have adapted this approach to control engineered T-cells through the use of a multichain receptor.

Here, we describe a strategy to create a switchable engineered CAR T-cells. Our approach is based on engineering a system that is directly integrated in the hinge domain that separate the scFv from the cell membrane. In addition, we chose to implement this strategy in a novel CAR architecture that relies on the FceRI receptor scaffold.

The particularity of this design resides in the possibility to split or combine different key functions of a CAR such as activation and costimulation within different chains of a receptor complex, mimicking the complexity of the TCR native architecture. In this report, we showed that the hinge engineering approaches allowed to turn a T-cell endowed with an engineered CAR from an off-state to an on-state.

By controlling the scFv presentation at the cell surface upon addition of the small molecule, our system allowed to further induce the cytolytic properties of the engineered T-cell. Overall, this non-lethal system offers the advantage of a "transient CAR T-cell" for safety while letting open the possibility of multiple specific cytotoxicity cycles using a small molecule drug.

Principles of T Cell Engineering and CAR Design

(A) Integration of B cell and T cell antigen recognition principles in the design of CARs. The heavy and light chain chains, which are components of the B cell receptor and Igs, are fused to the T-cell-activating z chain of the TCR-associated CD3 complex to generate non-MHC restricted, activating receptors capable of redirecting T cell antigen recognition and cytotoxicity.

(B and C) Integration of T cell activation and costimulation principles in dual signaling CARs designed to enhance T cell function and persistence in addition to retargeting T cell specificity. In

(B), the physiological abTCR associated with the CD3 signaling complex is flanked by the CD28 costimulatory receptor.

(C) shows a prototypic second-generation CAR, which comprises three canonical components: an scFv for antigen recognition, the cytoplasmic domain of the CD3z chain for T cell activation, and a costimulatory domain to enhance T cell function and persistence. Unlike the abTCR/CD3 complex, which comprises g, d, ε, and z signaling chains and is modulated by a multitude of costimulatory receptors, CARs possess in a single molecule the ability to trigger and modulate antigen-specific T cell functions.

9.2 Generational Architecture

There are currently three generations of CAR T cell design. We examine each here. As Cartellieri et al note:

In an attempt to extend the recognition specificity of T lymphocytes beyond their classical MHC-peptide complexes, a gene-therapeutic strategy has been developed that allows redirecting T cells to defined tumor cell surface antigens. This strategy uses both the cellular and humoral arm of the immune response by assembling an antigen-binding moiety, most commonly a single chain variable fragment (scFv) derived from a monoclonal antibody, together with an activating immune receptor.

Once this artificial immune receptor is expressed at the surface of a modified T lymphocyte, upon binding of the scFv to its antigen an activating signal is transmitted into the lymphocyte, which in turn triggers its effector functions against the target cell (Figure 2). In the first attempts to reconfigure T cells with antibody specificity the variable parts of the TCR α and β chains were replaced with scFv fragments derived from monoclonal antibodies. These hybrid T-cell receptors were functionally expressed and recognized the corresponding antigens in a non-MHC-restricted manner. As a consequence of the finding, that CD3ζ chain signaling on its own is sufficient for T-cell activation, the first "true" chimeric single-chain receptors were created by fusing a scFv

directly to the CD3ζ chain. At that time this concept was called the "T body approach". Nowadays these types of artificial lymphocyte signaling receptors are commonly referred to as chimeric immune receptors (CIRs) or chimeric antigen receptors (CARs).

The use of CARs to redirect T cells specifically against TAA-expressing tumor cells has a number of theoretical advantages over classical T-cell-based immunotherapies. In contrast to the long-lasting procedure of in vitro selection, characterization, and expansion of T-cell clones with native specificity for MHC tumor peptide complexes, genetic modification of polyclonal T-cell populations allows to generate TAA-specific T cells in one to two weeks. Engraftment with CARs enables T cells to MHC-independent antigen recognition; thus, major immune escape mechanisms of tumors such as downregulation of MHC molecules are efficiently bypassed.

Furthermore, proliferation and survival of modified T cells can be improved by the implementation of a multitude of signaling domains from different immune receptors in a single CAR

9.2.1 First Generation

Following Cartellieri et al we note regarding all three generations that:

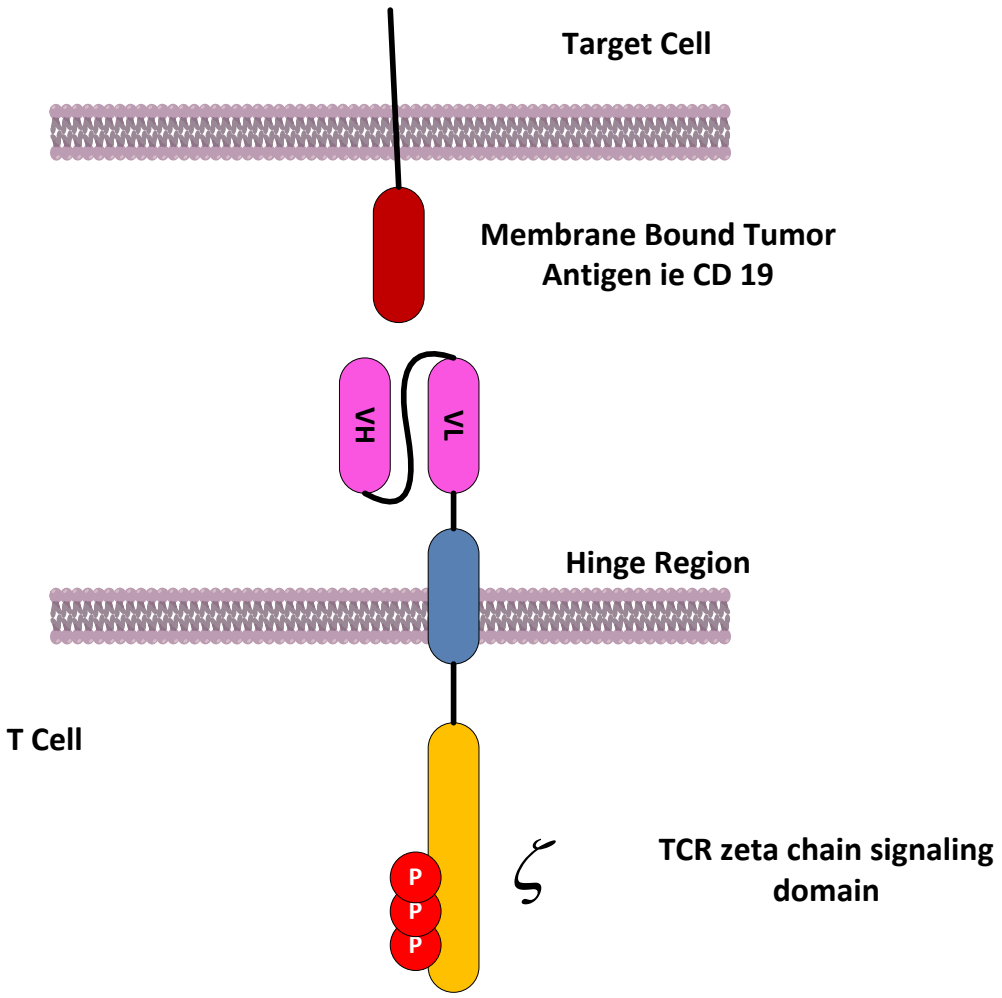
Evolution of CAR signaling capacities.

First generation CARs transmitted activating signals only via ITAM-bearing signaling chains like CD3ζ or FcεRIγ, licensing the engrafted T cells to eliminate tumor cells.

Second generation CARs contain an additional costimulatory domain (CM I), predominantly the CD28 domain. Signaling through these costimulatory domain leads to enhanced proliferation, cytokine secretion, and renders engrafted T cells resistant to immunosuppression and induction of AICD.

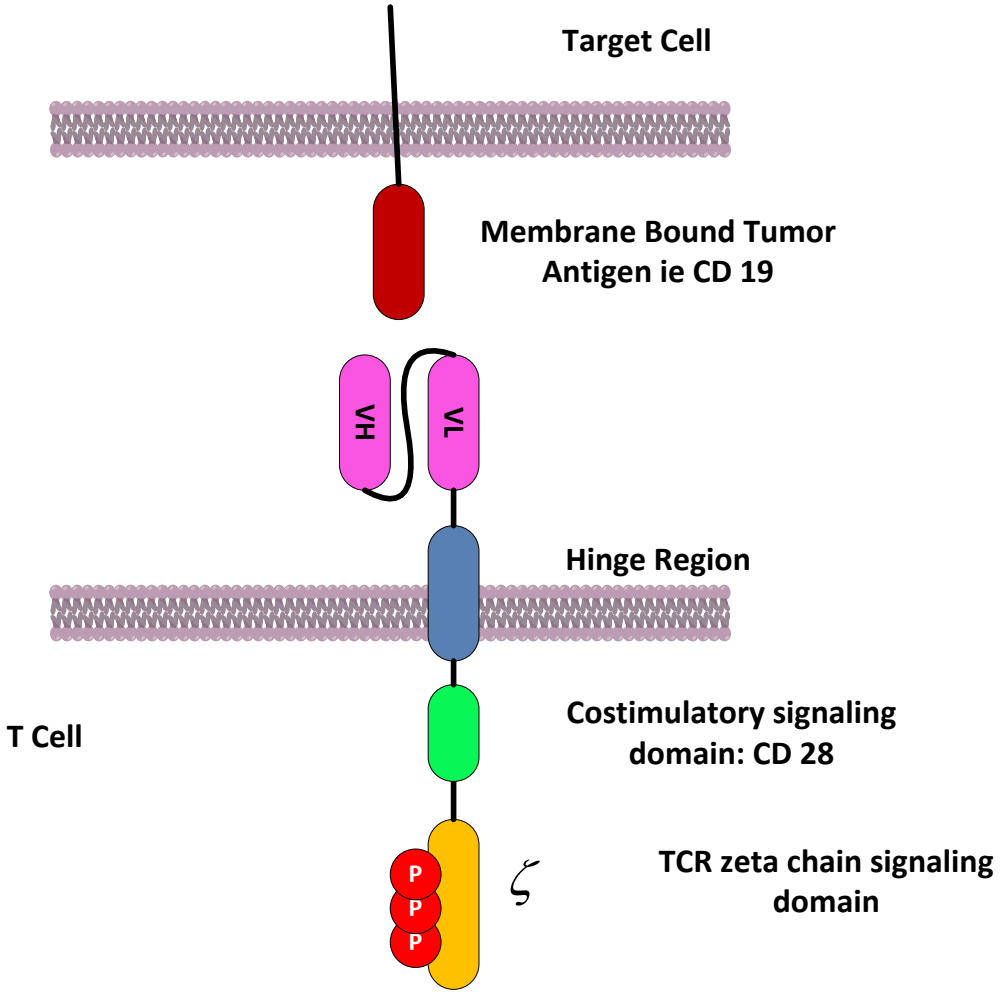
(Third Generation) Recent developments fused the intracellular part of a second costimulatory molecule (CM II) in addition to CD28 and ITAM-bearing signaling chains, thus generating tripartite signaling CARs. T cells engrafted with third generation CARs seem to have superior qualities regarding effector functions and in vivo persistence.

The first generation shown below is the simplest.



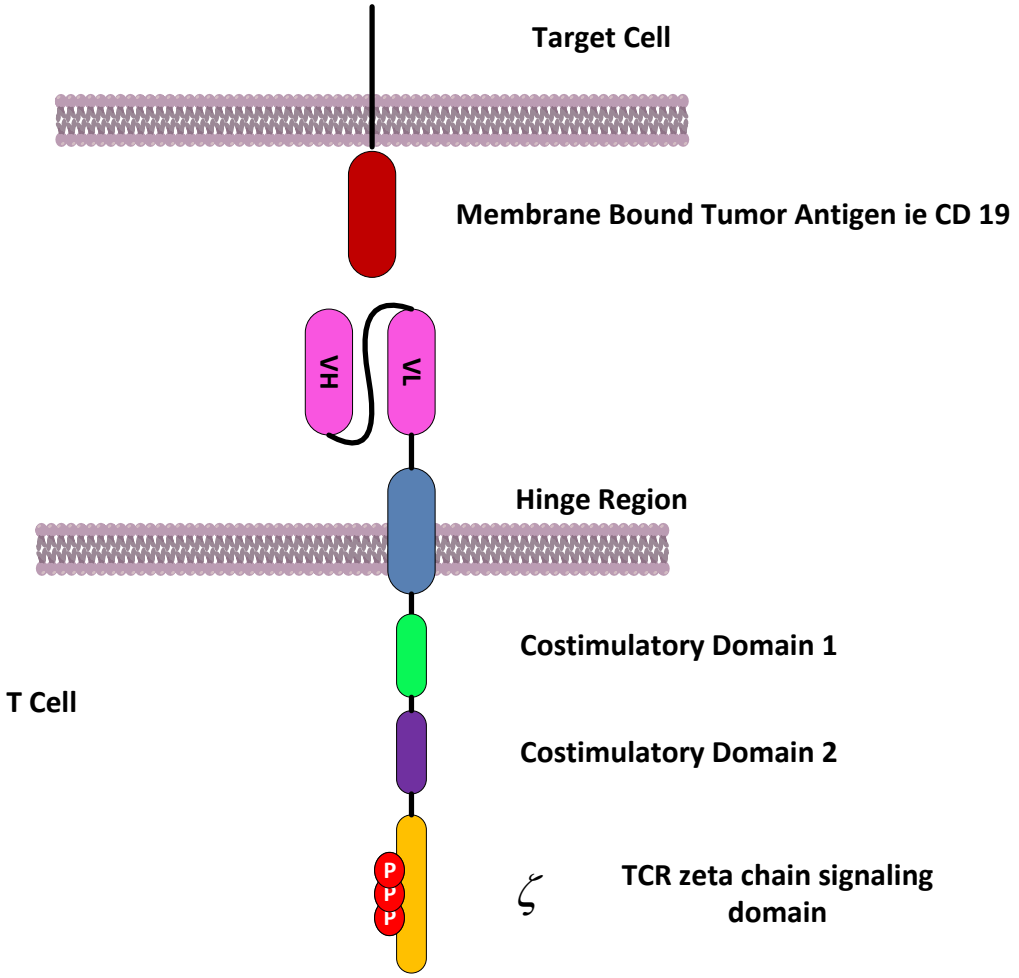
9.2.2 Second Generation

The second generation is as per below with the added element.



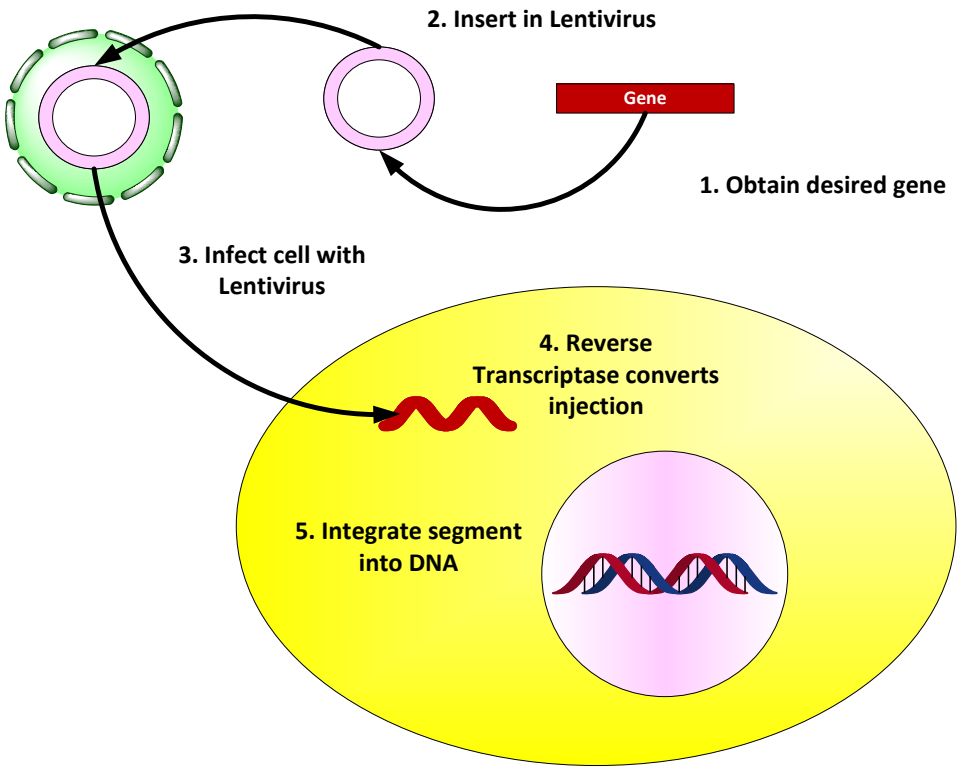
9.2.3 Third Generation

The third generation has added flexibility as shown below and described above.



9.3 Reverse Transcription and Gene Insertion

Now the insertion of the genes to create the previously described receptors uses a reverse transcription process. It is akin to what we see in HIV reverse transcription and specifically uses lentiviruses as the delivery mechanism.



As Naldini notes regarding lentiviruses:

Major hurdles for hematopoietic-stem-cell (HSC) gene therapy include achieving efficient ex vivo gene transfer into long-term repopulating HSCs, preventing activation of oncogenes by the nearby integration of a vector and controlling transgene expression to avoid ectopic or constitutive expression that leads to toxicity.

As compared to early generation gammaretroviral vectors (γ-RVs), HIV-derived lentiviral vectors result in more efficient gene transfer and stable, robust transgene expression in HSCs and their multilineage progeny. Extensive preclinical work indicated important features in vector biology and design that affect genotoxicity and highlighted strategies to alleviate it. The self-inactivating long terminal repeats (LTRs) and integration-site preferences of lentiviral vectors were shown to substantially alleviate insertional genotoxicity.

When tested in γ-RVs, the self-inactivating LTR design was shown to improve the safety of this platform as well. Retrospective analysis of several earlier trials suggests that disease background, transgene function, ex vivo culture and the efficiency of host repopulation can all influence the likelihood that insertional genotoxicity will manifest in a trial.

These data helped to shape the ideas that not all integrating vectors have the same effects and that genome-wide integration of improved vector designs, although still a mutagenic event, can be tolerated in the absence of aggravating circumstances. Self-inactivating lentiviral vectors are also being used to engineer T cells with chimeric antigen receptors (CARs) or T-cell antigen receptors for use in adoptive immunotherapy for the treatment of cancer. The advantages of this

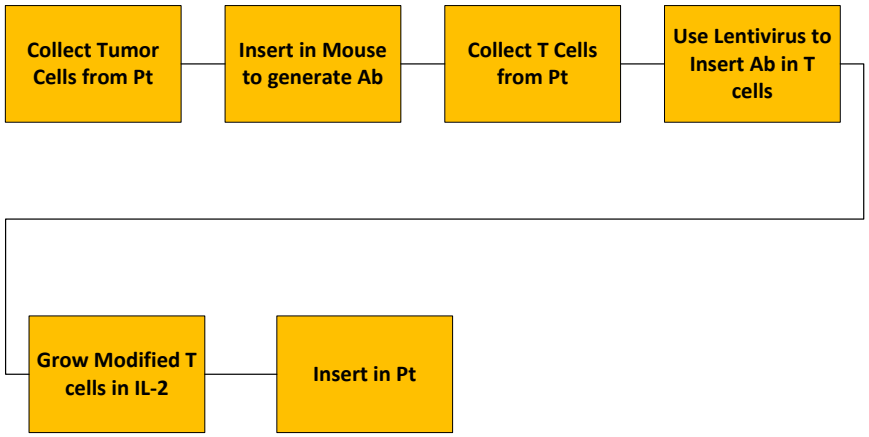
new platform in comparison to earlier-generation γ-RVs, which perform satisfactorily in this cell target, are yet to be fully established. Lentiviral vectors are thought to give rise to more robust and stable transgene expression in T cells in vivo, and could facilitate more efficient and versatile ex vivo gene transfer while supporting coordinated expression of multiple transgenes.

These advantages will become more relevant as the gene-therapy field implements refined strategies, such as improved T-cell manipulation to preserve T memory stem cells, or more demanding cell-engineering tasks, such as the co-expression of multiple CARs (to improve specificity) or a conditional safety switch/suicide gene (to improve safety).

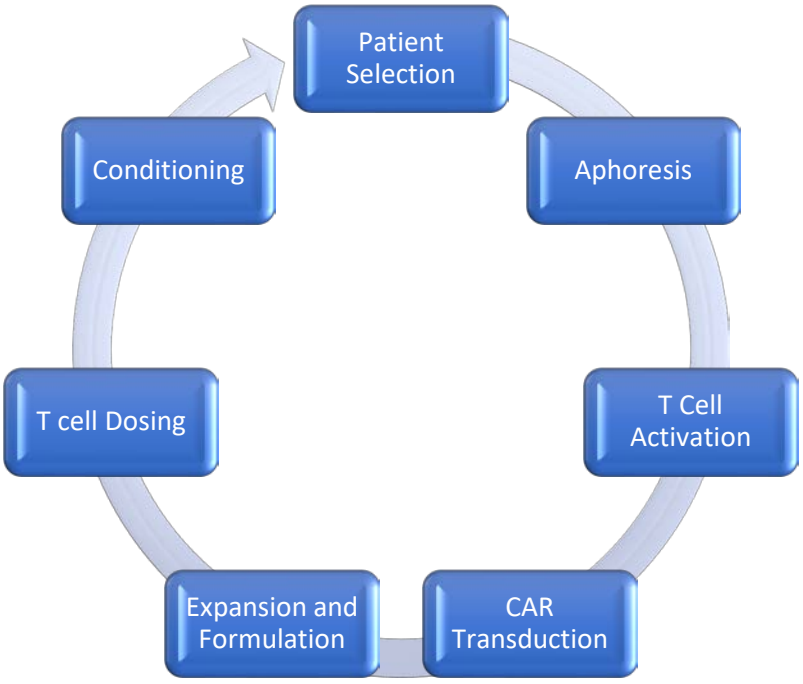
9.3.1 The Entire Process

We now review the process below. We have initially presented a logical approach, then we explained how it could be accomplished and now we return and demonstrate how this could be accomplished. We explain in detail in the Appendix a multiplicity of such protocols in use today.

9.3.2 Detailed Steps



We demonstrate below another view of this process. It may have to be an iterative one since certain initial CARs may handle the initial targets but as the tumor is subject to mutation we may have to adjust on an ongoing basis for new targets.



9.3.3 Switch Control

Now the mechanism above may lose some elements of control and switch mechanisms to turn it on or off have been considered.

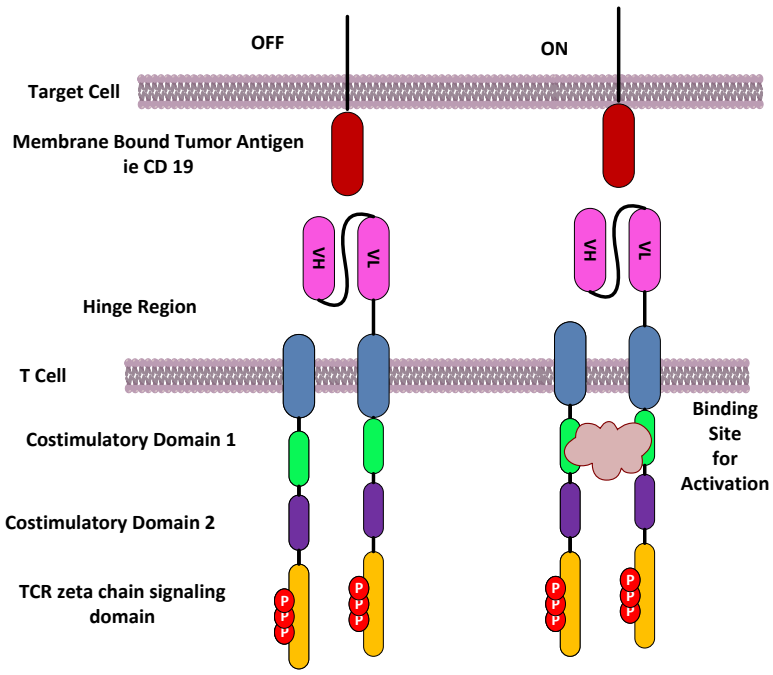
From Wu et al a specific mechanism is presented with its advantages and possible concerns. They state:

Cell-based therapies have emerged as a promising treatment modality for diseases such as cancer and autoimmunity. T cells engineered with synthetic receptors known as chimeric antigen receptors (CARs) have proven effective in eliminating chemotherapy resistant forms of B cell cancers. Such CAR T cells recognize antigens on the surface of tumor cells and eliminate them. However, CAR T cells also have adverse effects, including life threatening inflammatory side effects associated with their potent immune activity.

Risks for severe toxicity present a key challenge to the effective administration of such cell-based therapies on a routine basis.

The ON-switch CAR exemplifies a simple and effective strategy to integrate cell-autonomous decision-making (e.g., detection of disease signals) with exogenous, reversible user control. The rearrangement and splitting of key modular components provides a simple strategy for achieving integrated multi-input regulation. This work also highlights the importance of developing optimized bio-inert, orthogonal control agents such as small molecules and light, together with their cellular cognate response components, in order to advance precision-controlled cellular therapeutics.

We graphically demonstrate this mechanism below.



The authors continue:

Titratable control of engineered therapeutic T cells through an ON-switch chimeric antigen receptor. A conventional CAR design activates T cells upon target cell engagement but can yield severe toxicity due to excessive immune response.

The ON-switch CAR design, which has a split architecture, requires a priming small molecule, in addition to the cognate antigen, to trigger therapeutic functions. The magnitude of responses such as target cell killing can be titrated by varying the dosage of small molecule to mitigate toxicity. scFv, single-chain variable fragment; ITAM, immune receptor tyrosine-based activation motif.

9.4 OBSERVATIONS

CAR T cell therapy has had successes and failures. It seems to be appropriate for hematological cancers and some related ones where immunodeficiency is an element. However, it often has some several unintended consequences. The immune system is a very powerful system in the body. Setting CTLs loose to do what they do best can be at times very overpowering. In addition, the use of these systems without a means to throttle them back can present a danger to a wide selection of patients. We examine some of these issues as follows.

9.4.1 Damage

As Brudno1 and Kochenderfer have noted:

CAR T cells could damage tissues that express the antigen recognized by the CAR. This mechanism of toxicity can be minimized but not eliminated by an exhaustive search for expression of a targeted antigen on normal tissues during preclinical development of a CAR.

Examples of this mechanism of toxicity have been reported in the literature. In one study, 3 patients with metastatic renal cell carcinoma who received infusions of autologous T cells transduced with aCAR targeting carboxyanhydrase- IX experienced grade increases in alanine aminotransferase, aspartate aminotransferase, or total bilirubin.

Liver biopsies of affected patients revealed a cholangitis with a T-cell infiltration surrounding the bile ducts, and bile duct epithelial cells were unexpectedly found to express carboxy-anhydrase-IX.

A patient with metastatic colorectal cancer who received an infusion of autologous CAR T cells directed against the antigenERBB2 (Her-2/neu) experienced acute respiratory distress and pulmonary edema requiring mechanical ventilation. The patient subsequently died.

As Pegram et al note:

CD19-targeted chimeric antigen receptor (CAR) T cells are currently being tested in the clinic with very promising outcomes. However, limitations to CAR T cell therapy exist. These include lack of efficacy against some tumors, specific targeting of tumor cells without affecting normal tissue and retaining activity within the suppressive tumor microenvironment. Whilst promising clinical trials are in progress, preclinical development is focused on optimizing CAR design, to generate "armored CAR T cells" which are protected from the inhibitory tumor microenvironment. Studies investigating the expression of cytokine transgenes, combination therapy with small molecule inhibitors or monoclonal antibodies are aimed at improving the anti-tumor efficacy of CAR T cell therapy. Other strategies aimed at improving CAR T cell therapy include utilizing dual CARs and chemokine receptors to more specifically target tumor cells. This review will describe the current clinical data and some novel "armored CAR T cell" approaches for improving anti-tumor efficacy therapy.

9.4.2 Specific Damage

From FDA presentations, the following is a summary of such damages:

Tumor Lysis Syndrome
Cytokine Release Syndrome (CRS)
Organ specific toxicities

where:

Tumor Lysis Syndrome:

1. Urinary symptoms
2. Renal failure from elevated uric acid levels

3. Abdominal pain
4. Electrolyte abnormalities
    a. Hyperkalemia – weakness, cardiac rhythm abnormalities
    b. Hypocalcemia – cramps, tetany, cardiac rhythm abnormalities

CRS Clinical Manifestations:

1. Life-threatening
2. Hypotension
3. Fever
4. Hypoxia
5. Multi-organ failure
6. Coagulation disorders

9.5 Appendix: Production (Methods and Protocols)

We present summaries of two protocol descriptions.

9.5.1 Maude et al

From Maude et al in NEJM the following is an overview of their protocol:

1. *Peripheral blood mononuclear cells (PBMCs) were collected by leukapheresis,*

2. *T cells were enriched by mononuclear cell elutriation, washed,*

3. *and expanded by addition of anti-CD3/CD28–coated paramagnetic beads for activation of T cells.2*

4. *The lentiviral vector containing the previously described CD19-BB-ζ transgene2 (produced by the Vector Core at the Children's Hospital of Philadelphia) was added at the time of cell activation*[46]

5. *and was washed out on day 3 after culture initiation.*

6. *Cells were expanded on a rocking platform device (WAVE Bioreactor System) for 8 to 12 days.*

[46] Note there are many Vector Core programs where specific molecules can be obtained. For example
https://medicine.umich.edu/medschool/research/office-research/biomedical-research-core-facilities/vector
https://www.med.unc.edu/genetherapy/vectorcore
https://www.med.upenn.edu/gtp/vectorcore/
http://med.stanford.edu/gvvc/
https://sites.duke.edu/dvvc/
http://www.umassmed.edu/research/cores/viralvectorcore/

7. *On the final day of culture, the beads were removed by passage over a magnetic field and the CTL019 cells were harvested and cryopreserved in infusible medium.*

8. *Final product release criteria in the IND included the following:*

a. *cell viability ≥ 70%,*
b. *CD3+ cells ≥ 80%,*
c. *residual paramagnetic anti-CD3/CD28-coated paramagnetic beads ≤ 100 per 3x106 cells,*
d. *Endotoxin ≤ 3.5 EU/mL,*
e. *Mycoplasma negative,*
f. *Bacterial and fungal cultures negative,*
g. *residual bovine serum albumin ≤ 1 μg/mL,*
h. *VSV-G DNA as a surrogate marker for replication competent lentivirus ≤ 50 copies per μg DNA,*
i. *transduction efficiency by flow cytometry ≥ 2%, transduction efficiency by vector DNA sequence 0.02 to 4 copies per cell.*

9.5.2 Wu et al

Now from Wu et al in Science we have further details:

9.5.2.1 Construction of ON-switch CARs

The nucleotide sequence encoding a signal sequence, (was obtained as follows):

1. the anti-human CD19 antigen ligand binding scFv, and the human CD8a hinge and transmembrane domain …

2. Insertion of a Myc epitope tag immediately downstream of the signal sequence was performed by PCR.

3. The human 4-1BB co-stimulation and CD3 ITAM signaling chains were cloned from cDNAs …

4. Sequences encoding the FKBP and the T2089L mutant of FRB domains were obtained …

5. Sequences encoding the gibberellin-binding GID1 and GAI were also obtained …

6. The sequence encoding the anti-mesothelin scFv HN1 was synthesized by assembly PCR .

7. cloning techniques (PCR, restriction digestion, ligation, etc.) were applied to construct CAR expression plasmids using a second-generation self-inactivating lentiviral vector.

9.5.2.2 Culturing conditions for T cells and target cells

1. A Jurkat T cell line engineered with a NFAT-dependent EGFP reporter gene …

2. Jurkat T cells, Raji B cells and Daudi B cells were maintained in RPMI- 1640 medium supplemented with 10% FBS, penicillin and streptomycin.

3. K562 target cell lines were provided … cultured in IMDM medium supplemented with 10% FBS.

4. Cell density and average cell size (to help with assessing activated or resting

5. Primary human CD4+ or CD8+ T cells were isolated from anonymous healthy donor's blood after apheresis

6. Cells were enriched by negative selection

7. Isolated T cells were cryopreserved in RPMI-1640 medium supplemented with 20% human AB serum and 10% DMSO until use.

8. Two days prior to lentiviral transduction, cells were thawed and cultured in human T cell medium

9. Recombinant human IL-2 was added to a final concentration of 30 lU/mL for CD4+ cells and to 100 lU/mL for CD8+ T cells.

9.5.2.3 Lentiviral engineering of T cells and K562 target cells

1. Pantropic VSV-G pseudotyped lentivirus was produced from Lenti-X 293T cellsco-transfected with a pHR'SIN:CSW transgene expression vector and the viral packaging plasmids …using Lipofectamine

2. Infection medium/supernatant was collected 48 hours after transfection to transduce cells.

3. Twenty-four hours prior to viral transduction, primary human T cells were activated

4. Transduced primary T cells were maintained at ~106/mL in human T cell medium as previously described

5. Transduced Jurkat and K562 cells were cultured for at least 9 days before experiments were conducted. Expression of transgenes was confirmed by either staining with fluorophore-conjugated antibodies or by detecting fluorescent reporter proteins

9.5.2.4 Verifying CAR expression on T cells

1. Jurkat or primary human T cells were resuspended in FACS wash buffer (PBS + 0.5% BSA + 0.1% sodium azide) and stained with an Alexa 488- or Alexa 647- conjugated anti-myc antibody

2. Stained cells were washed BD Cytofix (BD #554655), and processed

3. FlowJo software (TreeStar) was used to quantify Alexa dye and/or mCherry fluorescence intensities.

4. Quantifying CD19 antigen ligand expression on target cells Target cells were pre-treated with human IgG in FACS wash buffer and then stained with an anti-human CD19-FITC antibody or isotype control

5. Stained cells were washed three times in wash buffer, fixed in a 1:1 mixture of the wash buffer.

6. FlowJo software was used to quantify FITC fluorescence intensity.

9.5.2.5 Quantitation of IL-2 and/or IFN-y production

1. Jurkat CD4+ T cells or primary CD4+ T cells expressing CARs were mixed with cognate (CD19+) or non-cognate (mesothelin+) K562 target cells at a 1:2 T cell target cell ratio.

2. The rapalog was serially diluted in medium and added to reaction mixtures.

3. Gibberellic acid acetoxymethyl ester was dissolved in ethanol and added to reaction mixtures.

4. After overnight incubation, supernatants were collected and analyzed with IL-2 or IFN-y ELISA

5. Flow cytometry was performed to quantify NFAT-dependent GFP reporter expression in Jurkat cells as a separate indicator for CAR activity.

9.5.2.6 Quantitation of CD69 surface expression

1. Primary CD4+ T cells expressing CARs were mixed with cognate (CD19+) or non-cognate (mesothelin+) K562 target cells at a 1:2 T celLtarget cell ratio in a U- bottom 96-well plate.

2. The rapalog A/C Heterodimerizer (Clontech Laboratories #635055) was serially diluted in medium and added to reaction mixtures.

3. After overnight incubation, cells were pelleted by centrifugation at 400g for 5min.

4. Cells were resuspended in FACS wash buffer (PBS + 0.5% BSA + 0.1% sodium azide) and stained with an Alexa 488 conjugated anti-human CD69 antibody (BioLegend #310916).

5. Stained cells were washed three times in FACS wash buffer, fixed in a 1:1 mixture of the wash buffer and BD Cytofix (BD #554655), and processed with a BD LSRII cytometer.

6. FlowJo software (TreeStar) was used to compare Alexa 488 fluorescence intensities of gated T cells (unique forward/side scatters) in samples.

9.5.2.7 Quantitation of T cell proliferation

1. Primary CD4+ T cells expressing the ON-switch CAR were labeled with CellTrace Violet (Life Technologies #C34557) following manufacturer's instructions. K562 target cells expressing either the cognate ligand (CD19) or non-cognate ligand (mesothelin) were treated with 25ug/mL mitomycin C (Molecular Toxicology, Inc. #60-100.20) for 30 minutes at 37°C to render target cells replication-incompetent.

2. T cells and target cells were mixed at 1:2 ratio, and the rapalog A/C Heterodimerizer (Clontech Laboratories #635055) was added to desired final concentrations.

3. Cells were collected daily for flow cytometry analysis (BD LSRII) after incubation for 3, 4, 5 and 6 days.

4. Histograms of CellTrace Violet stained cells were generated using FlowJo software (TreeStar).

5. Flow cytometry-based re-directed cytotoxicity/cell killing assay For Figure 4: The cognate (CD19+) and non-cognate (mesothelin+) K562 target cells were engineered with lentivirus to express the mCherry and GFP fluorescent proteins respectively, so that both cell types in a mixture could be simultaneously quantified by flow cytometry.

6. The two target cell lines were mixed at a 1:1 ratio and then co-incubated with resting primary human CD8+ T cells at 5:2 T celLtarget cell ratio in a U-bottom 96 well plate.

7. Human IL-2 was added to a final concentration of 100 lU/mL in each reaction well.

8. The hetero-dimerizing rapalog (Clontech Laboratories #635055) was added to concentrations noted in figures.

9. After intended periods of incubation, samples were centrifuged at 400g for 5 minutes.

10. Pelleted cells were resuspended in FACS wash buffer (PBS +0.5% BSA + 0.1% sodium azide) and fixed with an equal volume of BD Cytofix (BD #554655) prior to flow cytometry.

11. Control samples containing only the target cells were used to set a flow cytometry gate for intact target cells based on forward and side scatter patterns that had been previously confirmed to exclude apoptosed cells.

12. The gate was applied to all reaction samples, and abundance of the two target cell types was quantified.

13. A ratio of the surviving cognate target cells (mCherry+ GFP') to non-cognate target cells (mCherry' GFP+) was calculated for each sample to enumerate re-directed cytotoxic activities of T cells.

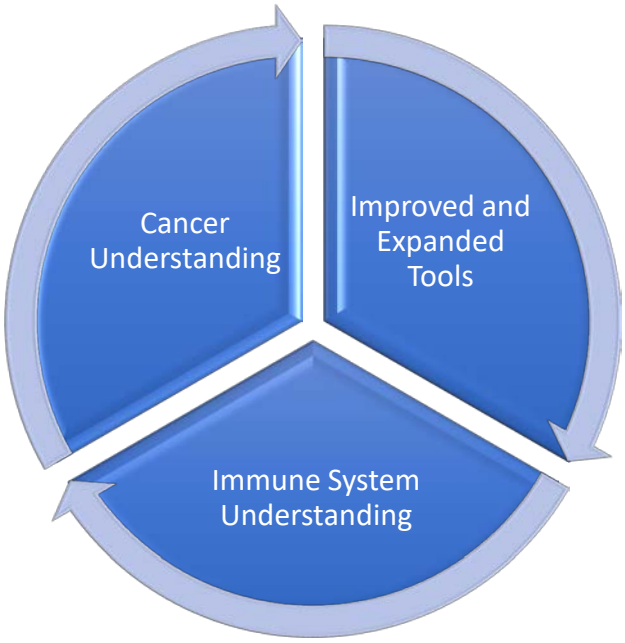
14. Flow cytometry data analysis was performed using FlowJo software (TreeStar).

15. Summary data plots were generated using Prism software (GraphPad).

10 IMMUNOTHERAPY OPTIONS

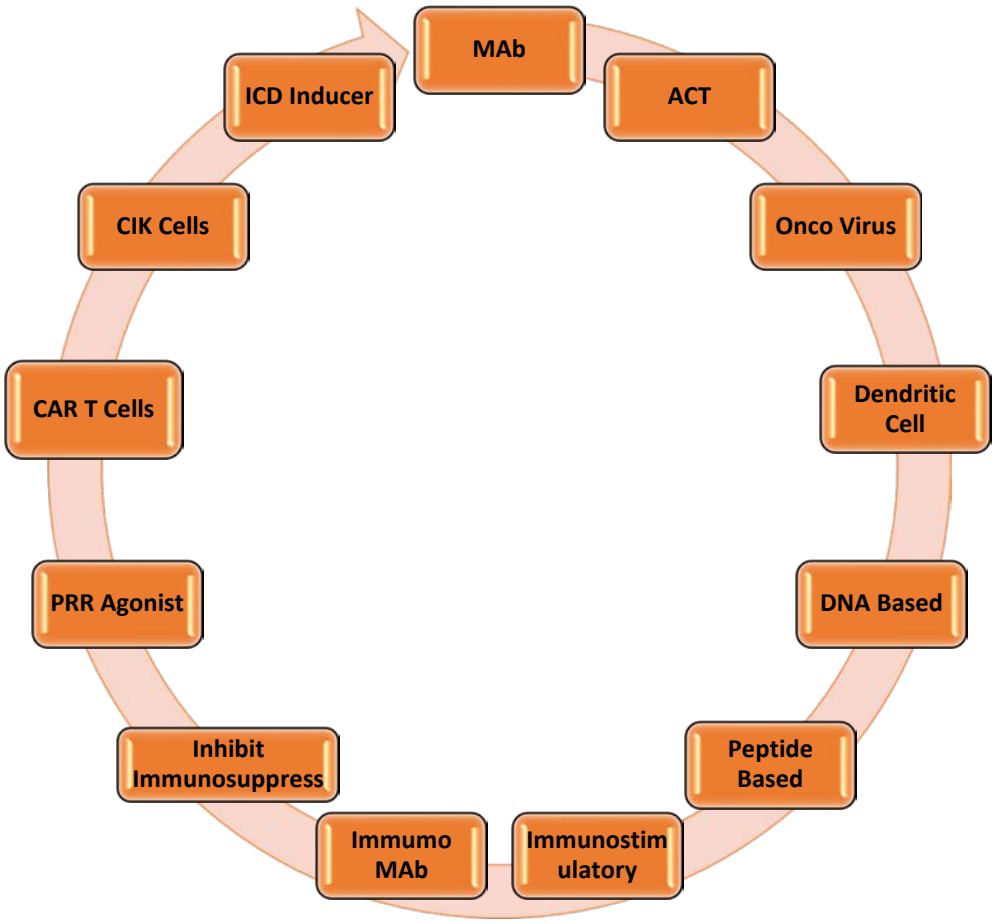
We now will examine the use of immunotherapeutic techniques in several areas. This is not an inclusive approach but a demonstrative one. It should be clear at this point that we are looking at a dynamically changing field and that what is said today will most likely change dramatically in a short period of time. The drivers are threefold:

1. Obtaining better tools
2. Understanding the immune system better
3. Understanding the specific cancer better



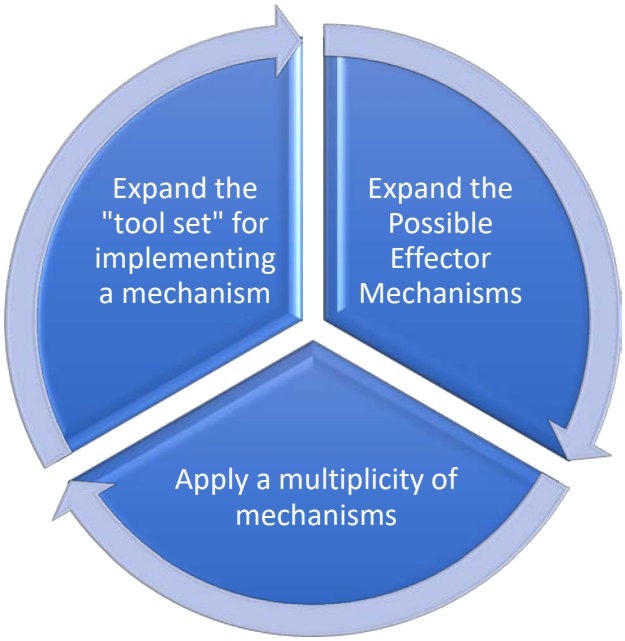
This three-part process is evolving at dramatic rates. Each area feeds the other and so forth. Thus, what we discuss here is at best a description of a point in time and not an exposition of how to accomplish tasks.

We use the Galluzzi et al classification as a useful tool in setting out the ways in which we can implement immunotherapy. It maps well onto the analysis we developed early on in the sense that it uses the tools available. We have added however another recent approach namely the CAR T cell. The chart below depicts the Galluzzi et al classifications as well as modifications containing recent updates.



The above demonstrates a variety of approaches in using immunotherapy. As we noted earlier, the immune system avails us with a set of cells, a set of inter cell communications mechanisms, a set of immune active proteins which can effect cell death and targeting.

In this sections we discuss a reasonable collection of the current approaches. However, it is possible to go in several directions beyond this. One is to enable alternative ways of using the core tool sets, another is combining multiple tools sets, and a third is to consider what can be achieved as we understand how to apply newer tool sets, such as what we have understood using CRISPR.



The above is a descriptive for thinking when examining the dimensions for development of immunotherapeutic options.

10.1 TUMOR-TARGETING MABS

As Galluzzi et al state:

Tumor-targeting mAbs are the best-characterized form of anticancer immunotherapy, and perhaps the most widely employed in the clinic. The expression "tumor-targeting" refers to mAbs that

(1) specifically alter the signaling functions of receptors expressed on the surface of malignant cells;

(2) bind to, and hence neutralize, trophic signals produced by malignant cells or by stromal components of neoplastic lesion;

(3) selectively recognize cancer cells based on the expression of a "tumor-associated antigen" (TAA), i.e., an antigen specifically (or at least predominantly) expressed by transformed cells but not (or at least less so) by their non-malignant counterparts.

Tumor-targeting mAbs exist in at least 5 functionally distinct variants.

First, naked mAbs that inhibit signaling pathways required for the survival or progression of neoplastic cells, but not of their non-malignant counterparts, such as the epidermal growth factor receptor (EGFR)-specific mAb cetuximab, which is approved by the US FDA for the treatment of head and neck cancer (HNC) and colorectal carcinoma (CRC).

Second, naked mAbs that activate potentially lethal receptors expressed on the surface of malignant cells, but not of their non-transformed counterparts, such as tigatuzumab (CS-1008), a mAb specific for tumor necrosis factor receptor superfamily, member 10B, (TNFRSF10B, best known as TRAILR2 or DR5) that is currently under clinical development.

Third, immune conjugates, i.e., TAA-specific mAbs coupled to toxins or radionuclides, such as gemtuzumab ozogamicin, an anti-CD33 calicheamicin conjugate currently approved for use in acute myeloid leukemia patients.

Fourth, naked TAA-specific mAbs that opsonize cancer cells and hence activate antibody dependent cell-mediated cytotoxicity (ADCC, antibody-dependent cellular phagocytosis, and complement-dependent cytotoxicity, such as the CD20-specific mAb rituximab, which is currently approved for the treatment of chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma.

Fifth, so-called "bispecific T-cell engagers" (BiTEs), i.e., chimeric proteins consisting of two single-chain variable fragments from distinct mAbs, one targeting a TAA and one specific for a T-cell surface antigen (e.g., blinatumomab, a CD19- and CD3 BiTE recently approved for the therapy of Philadelphia chromosome-negative precursor B-cell acute lymphoblastic leukemia).

10.2 ADOPTIVE CELL TRANSFER

From Galluzzi et al:

The term "adoptive cell transfer" (ACT) refers to a particular variant of cell-based anticancer immunotherapy that generally involves:

(1) the collection of circulating or tumor-infiltrating lymphocytes;

(2) their selection/ modification/expansion/activation ex vivo; and

(3) their (re-)administration to patients, most often after lymphodepleting pre-conditioning and in combination with immunostimulatory agents.

Other anticancer (immune)therapies involving the (re) infusion of living cells, such as hematopoietic stem cell transplantation (HSCT), conceptually differ from ACT. ACT involves the (re-)introduction of a cell population enriched in potentially tumor-reactive immune effectors.

HSCT is employed as a means to reconstitute a healthy, allogeneic (and hence potentially tumorreactive) immune system in patients with hematological malignancies previously subjected to myelo- and lymphoablating treatments (which aim at eradicating the majority of neoplastic cells). Dendritic cell (DC)-based interventions should also be conceptually differentiated from ACT for two reasons.

First, (re-) infused DCs are not endowed with intrinsic anticancer activity, but act as anticancer vaccines to elicit a tumortargeting immune response.

Second, DCs are not administered in the context of lympho/myeloablating chemo(radio)therapy

10.3 ONCOLYTIC VIRUSES

The term "oncolytic viruses" refers to nonpathogenic viral strains that specifically infect cancer cells, triggering their demise. Oncolytic viruses must be conceptually differentiated from so-called "oncotropic viruses", i.e., viruses that exhibit a preferential tropism for malignant cells but no (or very limited) cytotoxic activity. The antineoplastic potential of oncolytic viruses can be innate and simply originate from the socalled cytopathic effect, i.e., the lethal overload of cellular metabolism resulting from a productive viral infection.

As an alternative, these viruses can mediate an oncolytic activity because of (endogenous or exogenous) gene products that are potentially lethal for the host cell, irrespective of their capacity to massively replicate and cause a cytopathic effect. Of note, genetic engineering has been successfully employed to endow oncolytic virus with various advantageous traits, including sequences coding for

(1) enzymes that convert an innocuous pro-drug into a cytotoxic agent;

(2) proteins that (at least theoretically) trigger lethal signaling cascades in cancer cells only; or

(3) shorthairpin RNAs that target factors that are strictly required for the survival of transformed, but not normal cells. Of note, no oncolytic virus has been approved by the US FDA for use in cancer patients.

Conversely, a recombinant adenovirus (H101, commercialized under the name of Oncorine®) has been approved by the regulatory authorities of the People's Republic of China for the treatment of HNC (in combination with chemotherapy) as early as in November 2005. As oncolytic viruses are endowed with intrinsic anticancer activity, they are generally viewed as passive immunotherapeutics.

Moreover, several effectors of innate and adaptive immunity limit the efficacy of oncolytic therapy because they can neutralize viral particles before they reach neoplastic lesions. This is particularly true for the mononuclear phagocytic system of the liver and spleen, which is able to sequester large amounts of oncolytic viruses upon injection; the complement system, to which oncolytic viruses are particularly sensitive; and neutralizing antibodies, which can exist in patients prior to oncolytic virotherapy owing to their exposure to naturally occurring variants of the viral strains commonly employed for this purpose.

This being said, accumulating preclinical and clinical evidence indicates that the therapeutic activity of oncolytic viruses stems, for the most part, from their ability to elicit tumor-targeting immune responses as they promote the release of TAAs in an immunostimulatory context. In support of this notion, oncolytic viruses engineered to drive the expression of co-stimulatory

receptors or immunostimulatory cytokines/chemokines reportedly mediate superior antineoplastic effects as compared to their unmodified counterparts. Thus, conventional oncolytic viruses also appear to be active, rather than passive, immunotherapeutics.

10.4 DENDRITIC CELL BASED INTERVENTIONS

We often think of the DC as scavenger cells wandering around and picking up antigens and bringing them back for subsequent inspection and action. However, they can become active players as well in the therapeutic line. The Dendrite Cells are a key intermediary in the immune system exploring and delivering information of what invaders are where. Again, referring to Galluzzi et al they note:

Several forms of DC-based immunotherapy have been developed, most of which involve the isolation of patient- or donor-derived circulating monocytes and their amplification-differentiation ex vivo, invariably in the presence of agents that promote DC maturation, such as granulocyte macrophage colony-stimulating factor (GM-CSF). This is particularly important because immature DCs exert immunosuppressive, rather than immune stimulatory, functions.

*Most often, autologous DCs are re-infused into cancer patients upon exposure to a source of TAAs (**tumor associated antigens**), including*

(1) TAA derived peptides;

(2) mRNAs coding for one or more specific TAAs;

(3) expression vectors coding for one or more specific TAAs;

(4) bulk cancer cell lysates (of either autologous or heterologous derivation);

(5) or bulk cancer cell-derived mRNA.

As an alternative, DCs are allowed to fuse ex vivo with inactivated cancer cells, generating so called dendritomes. The rationale behind all these approaches is that DCs become loaded ex vivo with TAAs or TAA-coding molecules, hence becoming able to prime TAA-targeting immune responses upon reinfusion.

Additional DC-based anticancer immunotherapies include the targeting of specific TAAs to DCs in vivo, the use of DC-derived exosomes, and the (re-)administration of autologous or allogeneic DCs amplified, matured and optionally genetically modified ex vivo, but not loaded with TAAs. In the former setting, TAAs are fused to mAbs, polypeptides or carbohydrates that selectively bind to DCs, encapsulated in DC-targeting immunoliposomes, or encoded by DC-specific vectors. In the latter scenarios, DCs or their exosomes are administered as a relatively non-specific immunostimulatory intervention.

Interestingly, one cellular product containing a significant proportion of (partially immature) DCs is currently licensed for use in cancer patients, namely sipuleucel-T. Sipuleucel-T has been

approved by the US FDA and the EMA for the therapy of asymptomatic or minimally symptomatic metastatic castration-refractory prostate cancer as early as in 2010.

As Raval et al note:

Professional antigen presenting cells include Dendritic Cells (DCs), Macrophages, and B-cells. Of these DCs are the most potent antigen presenters given their morphologic and phenotypic properties. DCs in the skin were initially discovered by Paul Langerhans and were termed dendritic cells by Ralph Steinman due to the numerous dendrites which serve to increase the surface area for antigen presentation and cell-cell interactions.

Important for their function, these dendrites facilitate high concentrations of MHC-antigen complexes and cell surface co-stimulatory molecules required for robust T-cell activation. In this way, DCs serve as a key link between the innate and adaptive arms of the immune system. DCs can develop from either myeloid or lymphoid hematopoietic lineages, which can thus give rise to different subsets of DCs with varying functions.

Furthermore, DCs can have different effector functions depending on their tissue of residence and microenvironment. Langerhans cells are a subset of DCs which reside in the epidermal layers of the skin and function to continuously patrol and scan for pathogens. Langerin negative dermal DCs are a subset residing in the dermis and also play a key role in generating cellular immunity. Interstitial CD14+ DCs are thought to be less efficient at activating naïve T-cells and have proven tolerogenic functions

10.5 DNA-BASED VACCINES

Galluzzi states:

DNA-based anticancer vaccines rely on TAA coding constructs, be them naked or vectored (by viral particles, non-pathogenic bacteria or yeast cells). DNA-based vaccines either become a source of such TAA (as it is the case for bacterial and yeast vectors) or transform APCs or muscular cells to do so (as it is the case for naked constructs and viral vectors). Theoretically, and especially in the presence of adequate adjuvants, this prompts resident DCs or other APCs to prime a TAA-targeting immune response A particularly interesting approach in this context is represented by so-called "oncolytic vaccines", i.e., oncolytic viruses genetically altered to code for a TAA.

Promising results have also been obtained with DNA-based vaccines administered per os. In this setting, live-attenuated bacteria expressing a full length TAA are taken up by APCs in the intestinal mucosa, resulting in the priming of a robust, TAA-specific immune response in the so-called "mucosa-associated lymphoid tissue"

10.6 PEPTIDE-BASED VACCINES

…cytokines regulate (via autocrine, paracrine or endocrine circuits) virtually all biological functions. It is therefore not surprising that various attempts have been made to harness the

biological potency of specific cytokines to elicit novel or reinvigorate pre-existent tumor-targeting immune responses. The administration of most immunostimulatory cytokines to cancer patients as standalone therapeutic interventions, however, is generally associated with little, if any, clinical activity.

Thus, immunostimulatory cytokines are generally employed as adjuvants for other anticancer (immuno)therapeutics, either as recombinant molecules or encoded within expression vectors. Notable exceptions include interferon (IFN)-α2b (also known as Intron A®), and IL-2, which mediate single agent therapeutic activity in patients affected by melanoma, a tumor type particularly sensitive to immunotherapy. IFN- α2b is currently approved by the US FDA and EMA for the therapy of hairy cell leukemia (HCL), AIDS related Kaposi's sarcoma, follicular lymphoma, multiple myeloma, melanoma, external genital/perianal warts (condylomata acuminata) and cervical intraepithelial neoplasms (both as a recombinant, unmodified protein, and as a pegylated variant), while IL-2 is licensed for the treatment of metastatic forms of melanoma and RCC.

Moreover, IFN-α2a is approved for use in subjects with HCL and chronic phase, Philadelphia chromosome-positive chronic myeloid leukemia, upon minimal pretreatment (within 1 year of diagnosis). In Europe, IFN-α2a is also licensed for the treatment of melanoma.

Of note, GM-CSF and granulocyte colony-stimulating factor are approved by the US FDA and EMA for use in humans, but not as part of anticancer regimens. Nonetheless, GM-CSF has been shown to potentiate the clinical activity of several immunotherapeutics, including (but not limited to) peptide-based vaccines and immunomodulatory mAbs.

Recombinant tumor necrosis factor α (TNFα) is also licensed by several regulatory agencies worldwide (but not by the US FDA), for the treatment of limb-threatening soft tissue sarcoma and melanoma.

10.7 IMMUNOSTIMULATORY CYTOKINES

Cytokines are a normal part of the immune system and play various roles. Thus, it is natural to try and use them in some manner to either directly or indirectly attack tumor cells. For example, Interferon, has been used in the treatment of melanoma with varying results (See Tsao et al)

As Galluzzi et al state:

Taken as a family, cytokines regulate (via autocrine, paracrine or endocrine circuits) virtually all biological functions. It is therefore not surprising that various attempts have been made to harness the biological potency of specific cytokines to elicit novel or reinvigorate pre-existent tumor-targeting immune responses.

The administration of most immunostimulatory cytokines to cancer patients as standalone therapeutic interventions, however, is generally associated with little, if any, clinical activity. Thus, immunostimulatory cytokines are generally employed as adjuvants for other anticancer (immuno)therapeutics, either as recombinant molecules or encoded within expression vectors.

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However, in this setting TNFα is not employed as an immunostimulatory agent but administered in combination with melphalan (an alkylating agent) to increment the local concentration of the drug (and hence boost its cytotoxicity), and to promote the selective destruction of the tumor vasculature.

10.8 IMMUNOMODULATORY MABS

As Raval et al state:

Monoclonal antibodies (mAb) directed against tumor associated antigens like CD20 and HER-2 are a standard of care treatment in many malignancies. This technology was facilitated by the simultaneous understanding of antibody structure and the application of hybridoma technology, leading to a Nobel Prize for Jerne, Kohler, and Milstein in 1984. Antibodies are highly specific agents, and knowledge of their structure and potential modifications plays an increasingly important role in cancer immunotherapy.

There is an extremely diverse but highly specific region (with potentially low nanomolar affinity) called the Fab region which binds antigen fragments. In addition, the Fc region (constant domain) controls the host immune response. Though there are a variety of antibody subtypes, in the context of IgG antibodies (used primarily for therapeutics in oncology) there are four Fc gamma receptors (FcγR) in humans.

In general, the Fc portion of an antibody can interact with Fc receptors on cells such as natural killer (NK) cells, thus promoting mAb bound target cell lysis through a process known as antibody-dependent cellular cytotoxicity (ADCC). Monoclonal antibodies can also mediate

complement-dependent cytotoxicity (CDC) by directly activating the complement cascade and membrane attack complex (MAC).

CDC generally requires antibody crosslinking, and this approach is rarely used in the development of mAbs for cancer therapy. IgG1 mAb subtypes can often have the most significant ADCC, whereas mAbs of the human IgG4 subtype are thought to function primarily as agonists (signaling) or antagonists (blocking) with minimal ADCC, especially if Fc region glycoslyation is eliminated. Over the past few years a number of modified antibody technologies have emerged, including radioimmunotherapies, TRAP molecules, antibody-drug conjugates (ADCs), single chain dual specificity bi-specific T cell engagers (BiTEs), and chimeric antigen receptors (CARs).

Indeed, as early as 2002 the FDA approved radioimmunoisotopes to treat refractory non-Hodgkin's lymphoma with agents such as ibritumomab (anti-CD20 + Yttrium-90) and in 2003 tositumomab (anti-CD20 + Iodine 131). In 2012 the FDA approved the use of aflibercept (a TRAP molecule combining 2 separate regions mimicking VEGFR1 and VEGFR2 bound to an IgG1 Fc region) for metastatic colorectal carcinoma. There has also been a great deal of excitement about the development of antibody-drug conjugates, as these agents are designed to improve local delivery of highly toxic chemotherapeutics while simultaneously attempting to minimize systemic toxicity.

Now as Galluzzi et al state:

At odds with their tumor-targeting counterparts, immunomodulatory mAbs operate by interacting with (hence altering the function of) soluble or cellular components of the immune system. Thus, immunomodulatory mAbs are designed to elicit a novel or reinstate an existing anticancer immune response.

So far, this has been achieved through four general strategies:

(1) the inhibition of immunosuppressive receptors expressed by activated T lymphocytes, such as cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programmed cell death 1 (PDCD1, best known as PD-1), or NK cells, like various members of the killer cell immunoglobulin-like receptor (KIR) family;

The KIR are the receptors on the NK cells that allow them to recognize the presence of a putative pathogen. It is the innate immune system function similar to what is seen in the adaptive system, but in a more sophisticated manner. In addition, the CTLA-4 and PD-1 receptors are potentially inhibitors to the immune process and thus one way to get around this is to find a means to block their inhibiting function.

(2) the inhibition of the principal ligands of these receptors, such as the PD-1 ligand CD274 (best known as PD-L1 or B7-H1);

(3) the activation of co-stimulatory receptors expressed on the surface of immune effector cells such as tumor necrosis factor receptor superfamily, member 4 (TNFRSF4, best known as OX40),

TNFRSF9 (best known as CD137 or 4-1BB) [58, 314, 315], and TNFRSF18 (best known as GITR) and

(4) the neutralization of immunosuppressive factors released in the tumor microenvironment, such as transforming growth factor β1 (TGFβ1). The first of these approaches, which is commonly referred to as "checkpoint blockade", has been shown to induce robust and durable responses in cohorts of patients with a variety of solid tumors.

As it stands, no less than three checkpoint-blocking mAbs are currently approved by international regulatory agencies for use in humans (source http://www.fda.gov): (1) the anti-CTLA4 mAb ipilimumab (Yervoy™), which was licensed by the US FDA for use in individuals with unresectable or metastatic melanoma… the anti-PD-1 mAb pembrolizumab (Keytruda™), which received accelerated approval by the US FDA for the treatment of advanced or unresectable melanoma patients who fail to respond to other therapies … and nivolumab, another PD-1- targeting mAb licensed by the Japanese Ministry of Health and Welfare for use in humans… Based on the results of a recently completed Phase III clinical trial demonstrating that nivolumab significantly improves the progression-free and overall survival of patients with BRAFWT melanoma, the approval of this mAb by the US FDA is expected within the next few months.

The safety and efficacy of ipilimumab, pembrolizumab, nivolumab and other checkpoint-blocking mAbs are being demonstrated in a steadily expanding panel of oncological indications. Of note, some co-stimulatory mAbs including urelumab and PF-0582566 (both of which target CD137) are also under clinical development, with promising results…

10.9 PRR AGONISTS

PRR or Pattern Recognition receptors are part of the innate system. The Toll Like Receptors, TLR of which there are some sixteen at current count, are one class of PRR. There functions of the PRR are twofold. First, they recognize the general characteristics of invaders. Second, the recognize detailed structural characteristics as well. For example, TLR 4 has the ability to recognize the lipo-poly-saccharide molecule on cell membranes of bacteria[47].

From Galluzzi et al we have:

Pattern recognition receptors (PRRs) are evolutionarily conserved proteins involved in the recognition of danger signals. PRRs include (but are not limited to) Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain containing (NOD)-like receptors (NLRs).

Recall that the Toll (German for "weird") like receptors often are targets used to identify bacteria amongst other pathogens. They are one of many such receptors.

TLRs are transmembrane enzymatically-inactive proteins expressed by most APCs, including monocytes, macrophages and DCs, as well as by some types of epithelial cells. NLRs are expressed by a variety of cell types, including various components of the innate and adaptive

[47] See Sompayrac, p 20.

immune system. Taken together, PRRs sense a wide panel of danger signals, including exogenous "microbe-associated molecular patterns" (MAMPs) like bacterial lipopolysaccharide (LPS) or muramyl dipeptide (MDP), and endogenous "damage-associated molecular patterns" (DAMPs), like the non-histone nuclear protein high-mobility group box 1 (HMGB1) and mitochondrial DNA.

The activation of various PRRs ignites a signal transduction cascade with potent pro-inflammatory outcomes, including the activation of NF-κB, and the secretion of immunostimulatory cytokines, like type I IFNs and TNFα. Moreover, PRR signaling favors the maturation of DCs as well as the activation of macrophages and NK cells. Besides being critical for the response of the host to viral and bacterial challenges, some PRRs play a key role in the (re)activation of anticancer immune responses by chemo-, radio- and immunotherapeutic interventions

10.10 CAR T CELLS

CAR T cells are an immunotherapeutic technique that has demonstrated efficacy in a wide variety of cancers, especially hematopoietic. From Dai et al,

CARs are recombinant receptors that typically target surface molecules. CARs are typically composed of an extracellular antigen-recognition moiety that is linked, via spacer/hinge and transmembrane domains, to an intracellular signaling domain that can include costimulatory domains and T-cell activation moieties.

CARs recognize unprocessed antigens independently of their expression of major histocompatibility antigens, which is unlike the physiologic T-cell receptors (TCRs). Hence, CAR T-cells can circumvent some of the major mechanisms by which tumors avoid major histocompatibility class (MHC)–restricted T-cell recognition such as the downregulation of HLA expression or proteasomal antigen processing, two mechanisms that contribute to tumor escape from TCR-mediated immunity.

Another feature of CARs is their ability to bind not only to proteins but also to carbohydrate, ganglioside, proteoglycan, and heavily glycosylated protein, thereby expanding the range of potential targets. CARs typically engage the target via a single chain variable fragment (scFv) derived from antibodies, although natural ligands (known as first-generation CARs) and Fabs fragment (Fab) selected from libraries have also been used. Individual scFvs derived from murine immunoglobulins are normally used.

10.11 CIK, CYTOKINE INDUCED KILLER CELLS

CIK cells are an limnologically enhanced stew of various killer cells such as NK and T cells which are intensified and then replaced into the patient. Like many patient specific approaches, it requires patient cells which are then intensified. It is a form of personalized immunotherapy. As Sangiolo states:

Cytokine-induced killer (CIK) cells are a heterogeneous subset of ex-vivo expanded T lymphocytes which present a mixed T-NK phenotype and are endowed with a MHC-unrestricted antitumor activity. The main functional properties of CIK cells may address some of the main limitations that are currently preventing the successful clinical translation of adoptive immunotherapy strategies. Clinically adequate quantities of immune effectors, sufficient for multiple adoptive infusions, may be obtained based on their relatively easy and inexpensive ex-vivo expansion starting from peripheral blood mononuclear cells.

The MHC-unrestricted tumor-killing is mainly based on the interaction between NKG2D molecules on CIK cells and MIC A/B or ULBPs molecules on tumor cells; it has been proved effective against several solid and hematological malignancies and does not require any HLA-restriction increasing the number of patients that might potentially benefit from such approach.

Finally, CIK cells present a reduced alloreactivity across HLA-barriers with important clinical implications for their potential use as alternative to conventional Donor Lymphocyte Infusions after allogeneic hemopoietin cell transplant with a reduced risk of GVHD.

10.12 Overall Strategy

The immune system is a somewhat understood system whereby a cell which is not "normal" has certain characteristics, which if recognizable, can be converted into signal sent to the elements of the immune system whereby these elements may be assembled to effect an attack on this aberrant intruder. Thus, a small piece of wood, a splinter, may pierce the skin, and it is recognized by a macrophage via a Toll Like Receptor, and it then sends out signals via Interferon, and the neutrophils in the circulating see these signals on the surface of the blood vessels and the neutrophils are then stopped and penetrate the vessel and assemble en masse near the splinter point and start releasing massive amounts of cytokines to attack what they see as an invader. It may be a bit of a brutal attack but as a first line offence it generally works.

What the above sequel depicts is a sophisticated and yet still somewhat understood process, a machine if you will, which can be employed by the body to attack unwanted intruders. Fundamentally what we have are:

1. Sensors: We have molecules which can detect the presence of an invader. The TLR are a simple example. The TLR are also an example that demonstrates that their very existence was poorly understood even twenty years ago, but these sensors exist.

2. Message Carriers: There are mechanisms whereby the sensors can communicate with the responders. Interferon, Interleukin, and other chemicals are used. However, they are not used bluntly, but in an elegant and specific manner whereby the subse1quent responders somehow know where and what to attack.

3. Responders: These are the cells which go on the attack such as the NK cells or Neutrophils.

4. Weapons: The responder cells have a wealth of weapons available such as the cytokines. The problem at time is that the weapons may be too excessive and do collateral damage to other normal cells. We have seen this with CAR T cell applications.

11 HEMATOLOGICAL CANCERS: MDS

MDS is a proto-malignant state where the causative factor putatively is methylation of hematopoietic control genes often along the myelo line from stem cells. The result is a proliferation of blasts and immature cells and often with an aberrant growth in one or more cell lines including erythrocytes and platelets.

The typical initial approach is to use one of two possible suppressors on DMNT1 which can facilitate methylation in new cells. The two most common DMNT1 inhibitors are azacitidine and decitabine. However, the doses used are often quite large and have some morbidity and sequellae. In a recent paper by Saunthararajah et al the authors note:

MDS is genetically heterogeneous. Thus, in the effort to identify pretreatment characteristics that predict response to therapy, there has been a focus on potential mutational predictors, but without a strong mechanistic rationale. From a mechanistic perspective, however, a minimum requirement for response is achievement of the intended molecular pharmacodynamic effect (DNMT1 depletion), whatever the disease mutations.

DNMT1 depletion is S-phase dependent and hence drug exposure time dependent: effectiveness meriting FDA approval was achieved when decitabine doses were reduced to less than 10% of those initially evaluated but administered more often (lower doses caused less toxicity that enabled more frequent administration).

A further decrease in this initial FDA-approved dose from 45 mg/m2/day to 20 mg/m2/ day and a further increase in frequency of administration (5 days every 4 weeks instead of 3 days every 6 weeks) doubled the overall response rate from 30% to 63%. Underscoring the importance of frequency of administration, giving this latter lower dose less often (3 days/28-day cycle) decreased overall responses to 23%.

Thus, a reduction actually improved response.

However, the authors of this paper demonstrate a collection of genes which we will discuss herein as being of significance in this disorder. They state:

Hence, especially for some subtypes of myeloid malignancy, there is a need for treatments that are not mediated through p53 and apoptosis (noncytotoxic treatments). Several groups have observed that terminal differentiation is induced in vitro when treating myeloid and other cancer cells with drugs or conditions that inhibit gene-silencing, chromatin-modifying enzymes (chromatin relaxation).

These differentiation- mediated cell-cycle exits, like those that occur during normal tissue differentiation, do not require p53 and are readily induced in p53/p16-null cancer cells. The same chromatin-relaxing conditions increase the differentiation of normal progenitors as well but, in contrast, increase self-renewal of NHSCs. The reasons for this cell context–dependent

response have been evaluated: differentiation is driven by relatively few master transcription factors.

Myeloid cancer cells express master myeloid differentiation– driven transcription factors (e.g., CEBPA and PU.1) at high levels, yet the target genes of these transcription factors are epigenetically silenced because of aberrant recruitment of silencing — instead of activating — chromatin- modifying enzymes to the transcription factors. Inhibition of silencing enzymes with drugs such as decitabine restores expression of numerous target genes of the transcription factors, including MYC antagonists (e.g., CEBPE and p27/ CDKN1B), that terminate proliferation.

Normal stem cells, on the other hand, express master stem cell transcription factors (e.g., HLF and HOXB4) and activate stem cell genes and stem cell fate in response to the same treatments (good therapeutic index).

The above focuses on the importance of CEBPA, HLF and PU.1. The authors also focus on GATA1 as well. These all are control elements in hematopoiesis. Now it is known that there may be methylation on CpG islands ahead of these genes and such methylation would result in suppression of the gene. Suppression would then result in loss of control and putatively a resulting unstable set of growth.

We will use this paper to examine several issues. Specifically:

1. The effect of methylation.

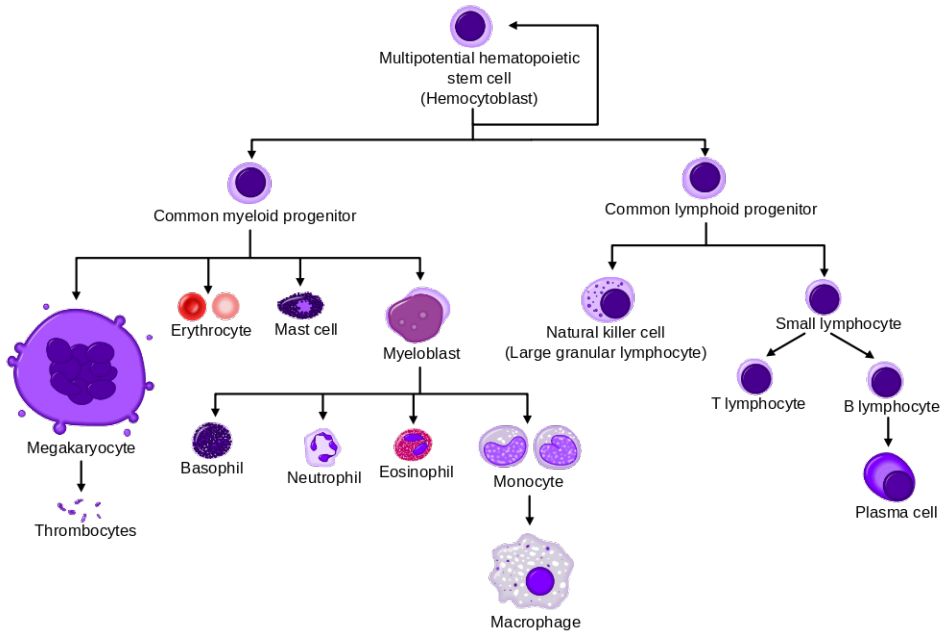
2. The location of methylation.

3. The cause of methylation.

4. The impact of suppressing DMNT1 and Thus, re-methylation of stem cell proliferates.

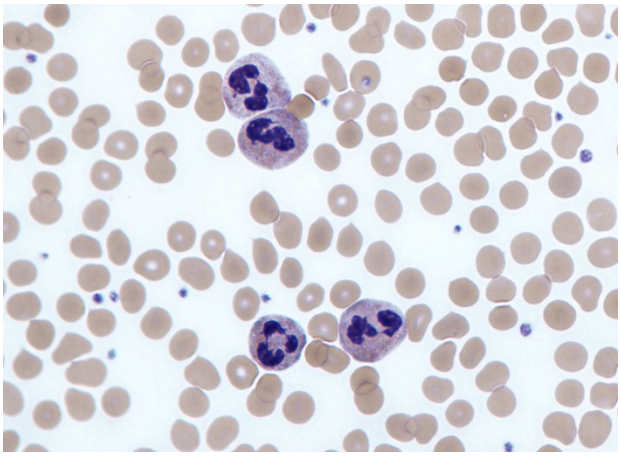
5. A re-analysis of the results of recent work using DNMT1 suppression followed by BMT and then the use of CIK cells. We had examined this in 2013 and results clinically have been favorable in several Phase 2 Trials.

Now when we examine MDS we often ask; when and where does the methylation occur. The following is the classic breakout of cells from the stem cell:



Now we often see low erythrocytes or thrombocytes (platelets) as well as low neutrophils. Low red cells result in an anemia and this is often one of the major presentations of MDS. Low platelets are the cause of poor clotting and excessively low platelets lead to catastrophic DIC. Low neutrophils result in possible uncontrolled infections including sepsis. Oftentimes the diagnosis of MDS is incidental to these and other findings.

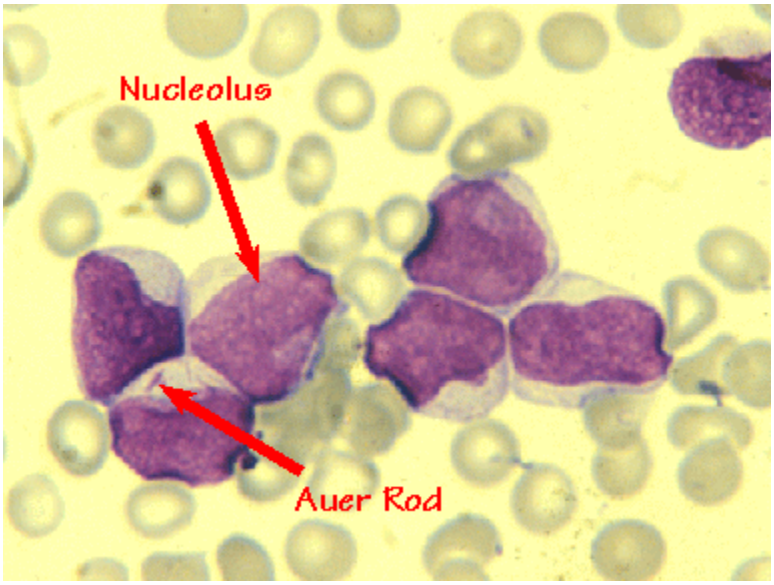
If we examine neutrophils we typically see the following:



The neutrophils are multilobed and mature is what one would expect in the circulatory system. Immature neutrophils, called bands, are often indicative of infection and blasts are very immature cells often indicative of an advanced MDS process.

One question is that in MDS we see just one line, say the myeloid line, showing the effects of methylation. However, the natural sequella to MDS is AML and this is a broad loss of control across the myeloid line.

Blast cells are immature precursors of either lymphocytes (lymphoblasts), or granulocytes (myeloblasts). They do not normally appear in peripheral blood. When they do, they can be recognized by their large size, and primitive nuclei (i.e. the nuclei contain nucleoli), as in the picture. When present in the blood, they often signify acute myelocytic leukemia, AML. This particular case below demonstrates the presence of an Auer Rod, which is pathognomonic for Acute Myeloid Leukemia. Otherwise, special stains and surface marker techniques are needed to identify the lineage of the cells.



As Pang et al note:

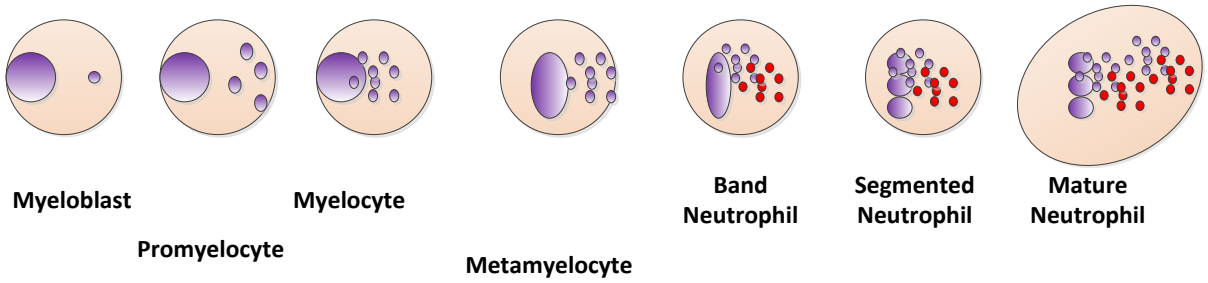
Myelodysplastic syndromes (MDS) are a group of disorders characterized by variable cytopenias and ineffective hematopoiesis. Hematopoietic stem cells (HSCs) and myeloid progenitors in MDS have not been extensively characterized. We transplanted purified human HSCs from MDS samples into immunodeficient mice and show that HSCs are the disease-initiating cells in MDS.

We identify a recurrent loss of granulocyte-macrophage progenitors (GMPs) in the bone marrow of low risk MDS patients that can distinguish low risk MDS from clinical mimics, Thus, providing a simple diagnostic tool. The loss of GMPs is likely due to increased apoptosis and increased phagocytosis, the latter due to the up-regulation of cell surface calreticulin, a prophagocytic marker.

Blocking calreticulin on low risk MDS myeloid progenitors rescues them from phagocytosis in vitro. However, in the high-risk refractory anemia with excess blasts (RAEB) stages of MDS, the GMP population is increased in frequency compared with normal, and myeloid progenitors evade phagocytosis due to up-regulation of CD47, an antiphagocyticmarker. Blocking CD47 leads to the selective phagocytosis of this population.

We propose that MDS HSCs compete with normal HSCs in the patients by increasing their frequency at the expense of normal hematopoiesis, that the loss of MDS myeloid progenitors by programmed cell death and programmed cell removal are, in part, responsible for the cytopenias, and that up-regulation of the "don't eat me" signal CD47 on MDS myeloid progenitors is an important transition step leading from low risk MDS to high risk MDS and, possibly, to acute myeloid leukemia.

Now if one looks at the neutrophil line one sees the progression from the myeloblast, blast, in the bone marrow, to the mature neutrophil in the blood stream. We demonstrate that below.



Now it is the presence of adequate neutrophils that result in a strong initial immune response. The failure of the marrow to allow maturation results in excess blasts and fewer mature neutrophils. That is one of the problems of having methylated areas around the genes forcing that maturation process.

There are three transcription factors that play a prominent role here. They are:

1. CEPBA
2. PU.1
3. GATA-1

Also discussed in Fiedler and Brunner are GATA-2. The authors state:

Several other studies dealing with certain aspects of the molecular interaction of PU.1 and GATA-1 as well as their gene regulatory capacity revealed the cross-antagonism between these proteins involving direct physical interaction of both factors that results in an inhibition of the transactivation trans-activation potential of the counterpart. Based on these findings, GATA-1 is prospected as the erythroid/megakaryocyte lineage determinant, whereas PU.1 is regarded as the myeloid/ lymphoid lineage determinant…..

Moreover, lineage choice between monocytes and granulocytes depends on the expression level of PU.1 and C/EBPα, which has been shown by studies using different mouse as well as in vitro models for diminished PU.1 expression in the hematopoietic system.

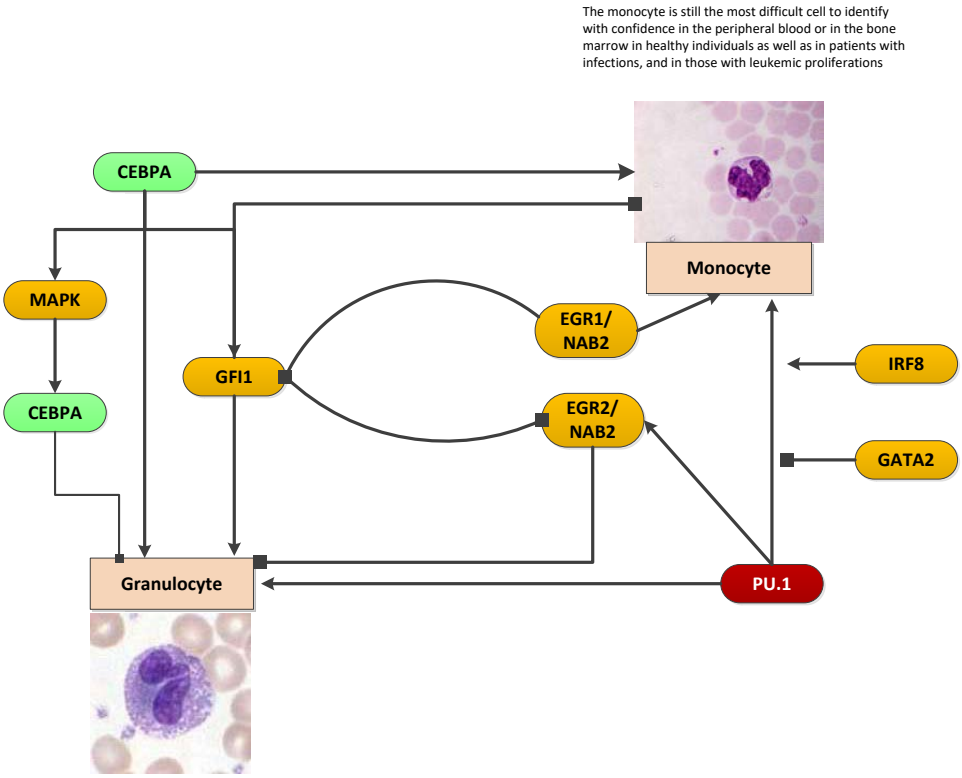
In all experimental setups, reduced expression of PU.1 is followed by an augmented granulopoiesis to the disadvantage of monocyte development. In line with these findings, we

have demonstrated that loss-of-function mutations of Btk in myeloid cells diminished the C/EBPα as well as PU.1 expression resulting consequently also in an increased granulopoiesis at the expanse of monopoiesis.

Additionally, gene expression analyses of PU.1-deficient progenitors revealed a decreased or even absent expression of several monocyte-specific genes, like the macrophage scavenger receptor or the M-CSF receptor….

Thus, for neutrophils there needs to be increased PU.1 and CEBPA to allow full maturation. It is Thus, expected that for lack to mature one must have an altered level. We will demonstrate that from the results in the focus paper.

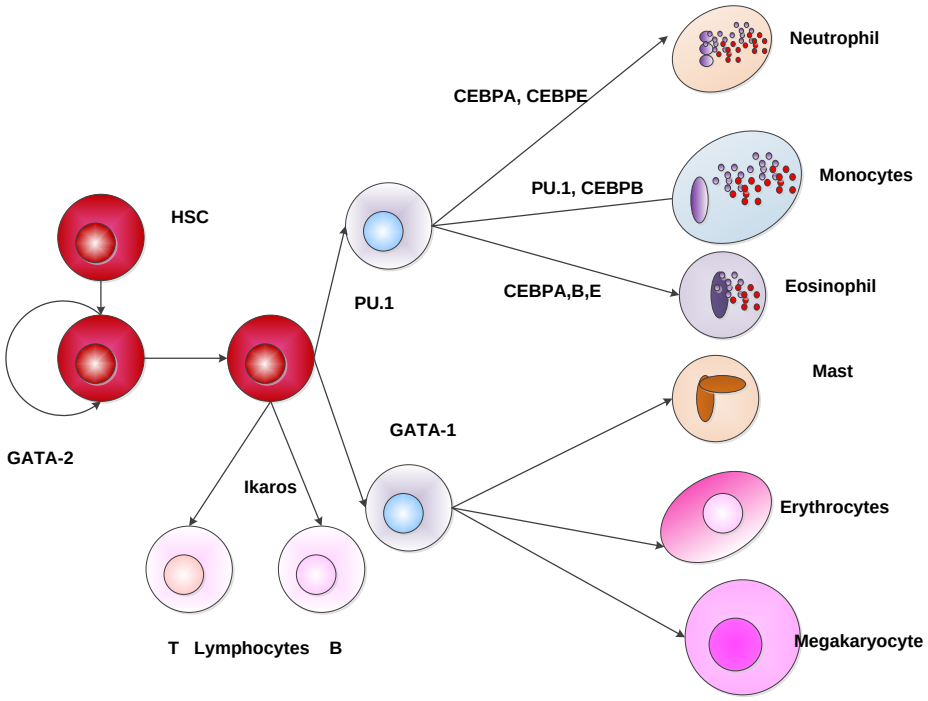
The management of monocyte to granulocyte is shown below with two of the genes we discuss herein. We return to this later.



11.1 Gene Expressions

We now will examine the genes referenced in the focus paper. Specifically, we examine HLF is a transcription factor and has been associated with leukemias. It also prolongs cell life and can be a facilitator for other transcription factors. CEBPA is another transcription factor gene and is a leucine zipper domain entity. It is well known that any mutations of this gene, or perhaps suppression by methylation, results in AML. CEBPA provides a control element is normal hematopoietic control. STI1 or PU.1 is another transcription factor which controls myeloid maturation as well as B cell maturation. We can often see the two names used interchangeably. GATA1 is also a transcription factor of the GATA family and plays a significant role in hematopoietic stasis.

The following is a descriptive from Hoffman et al (Modified Fig 25.3) regarding the active genes which are noted:



It should also be noted that the genes identified are transcription factors which activate genes which themselves are the operative genes. The above from Hoffman et al is indicative of the importance of these transcription factors.

Hoffman et al also there note:

Lineage-specific maturation of committed hematopoietic progenitor cells is ultimately driven by transcription factors, which have been hypothesized to be the final common pathway leading to commitment and differentiation of the pluripotent stem cell.

The role of transcription factors in cellular proliferation, differentiation, and survival of stem cells during hematopoiesis in the mammalian BM has been well established. Studies of the regulation of individual genes that show tissue- and stage-specific myeloid expression have implicated a small number of transcription factors that are responsible for directing both phenotypical myeloid maturation and the expression of functionally important myeloid genes. As described in detail subsequently, this role is underscored by the observations in AML, in which disruption of differentiation and defective myeloid-specific gene expression are linked to pathognomonic chromosomal translocations that result in the dysregulation of transcription factor expression.

Maturation of multipotent progenitor stem cells into specialized blood cells (lymphocytes, erythrocytes, neutrophils, monocytes, and eosinophils, among others) is regulated by a well-orchestrated interplay of transcription factors that are capable of instructing the expression of a specific set of genes within a specific lineage.

Here Hoffman et al emphasize the importance of the transcription factors. The question is however in the discussion on MDS; if we see an excess of these factors in the cells then there must be some other factor occurring and that factor is the methylation and effective blocking of the products of these genes, the transcription factor proteins, from doing what they were intended to do. Thus, a secondary protein or sets of proteins are not being produced and the resulting maturation is blocked.

Gene knock-out technology and overexpression studies, in conjunction with newer techniques that involve the use of multicolor fluorescence-activated cell sorting (FACS), have aided in delineating several transcription factors critical to the development of specific hematopoietic lineages. On the basis of these studies, critical transcription factors have been classified into two major categories. The first category includes factors such as stem cell leukemia transcription factor (SCL), GATA2, and AML factor-1 (AML-1) now known as Runx1, that influence differentiation to all of the hematopoietic lineages; the second category comprises the master regulators of lineage development, including GATA1, PU.1, and CCAAT enhancer–binding protein-α (C/EBPα).

These factors not only promote lineage-specific gene expression but also suppress alternative lineage pathways. (The Figure above) summarizes the postulated role of several key transcription factors during hematopoietic development. Myeloid progenitors exhibit multilineage patterns of gene expression. Studies by Laslo et al elegantly demonstrated that cell fate determination is dependent on subtle changes in expression levels of transcription factors, which regulate differential lineage maturation. For example, levels of PU.1 expression are increased by Egr-1/Nab-2 in developing macrophages; at the same time, Egr-1 represses the expression of the neutrophil specific Gfi-1 transcription factor, thereby simultaneously repressing the neutrophil development program

The following is a summary table of each of these genes. These are the genes examined as markers for the efficacy of reduce DMNT1 inhibitors in the MDS work by Sauntharajah et al. One of the key questions is; are these correct markers? A second question is: if they are then what caused their change in expression and how?

Gene	*Location*	*Function*
HLF[48]	17q22	This gene encodes a member of the proline and acidic-rich (PAR) protein family, a subset of the bZIP transcription factors. The encoded protein forms homodimers or heterodimers with other PAR family members and binds sequence-specific promoter elements to activate transcription. Chromosomal translocations fusing portions of this gene with the E2A gene cause a subset of childhood B-lineage acute lymphoid leukemias. Alternatively, spliced transcript variants have been described, but their biological validity has not been determined. It drives the hematopoietic stem cell fate.
CEBPA[49]	19q13.1	This intronless gene encodes a transcription factor that contains a basic leucine zipper (bZIP) domain and recognizes the CCAAT motif in the promoters of target genes. The encoded protein functions in homodimers and also heterodimers with CCAAT/enhancer-binding proteins beta and gamma. Activity of this protein can modulate the expression of genes involved in cell cycle regulation as well as in body weight homeostasis. Mutation of this gene is associated with acute myeloid leukemia. The use of alternative in-frame non-AUG (GUG) and AUG start codons results in protein isoforms with different lengths. Differential translation initiation is mediated by an out-of-frame, upstream open reading frame which is located between the GUG and the first AUG start codons.
STI1[50] also, PU.1	11p.11.2	This gene encodes an ETS-domain transcription factor that activates gene expression during myeloid and B-lymphoid cell development. The nuclear protein binds to a purine-rich sequence known as the PU-box found near the promoters of target genes, and regulates their expression in coordination with other transcription factors and cofactors. The protein can also regulate alternative splicing of target genes. Multiple transcript variants encoding different isoforms have been found for this gene.

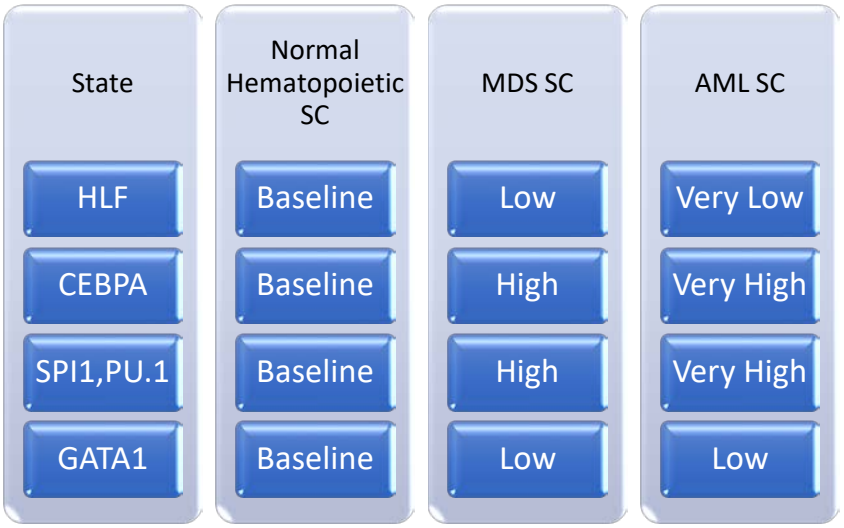
[48] http://www.ncbi.nlm.nih.gov/gene/3131

[49] http://www.ncbi.nlm.nih.gov/gene/1050

[50] http://www.ncbi.nlm.nih.gov/gene/6688

Gene	*Location*	*Function*
GATA1[51]	X.p.11.23	This gene encodes a protein which belongs to the GATA family of transcription factors. The protein plays an important role in erythroid development by regulating the switch of fetal hemoglobin to adult hemoglobin. Mutations in this gene have been associated with X-linked dyserythropoietic anemia and thrombocytopenia.

We can summarize the results of Sauntharajah et al in the following graphic as regards to these four genes. We show the normal baseline and then for MDS stem cells and then for Acute Myelogenous stem cell the expression of each of these genes relative to the normal state. Note the significant increases in CEBPA and PU.1 and the decrease in HLF. We will examine these in some detail. Our intent here is not to examine the work of Sauntharajah et al but to use it as a stepping off point for examining the issue of methylation. Namely what, where, and when are these sites methylated, if indeed that is the case.



As stated in the reference article by Sauntharajah:

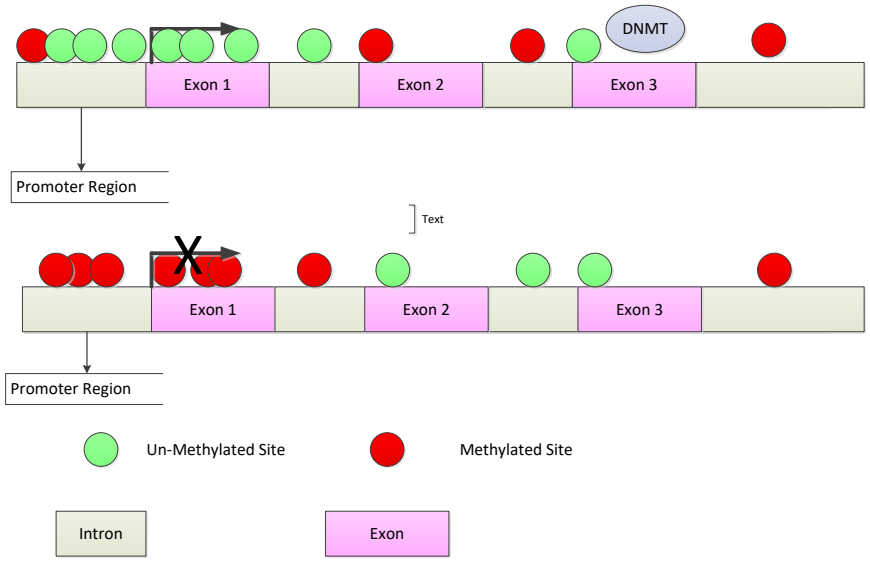
Myeloid cancer cells express master myeloid differentiation– driven transcription factors (e.g., CEBPA and PU.1) at high levels, yet the target genes of these transcription factors are epigenetically silenced because of aberrant recruitment of silencing — instead of activating — chromatin- modifying enzymes to the transcription factors.

Inhibition of silencing enzymes with drugs such as decitabine restores expression of numerous target genes of the transcription factors, including MYC antagonists (e.g., CEBPE and p27/ CDKN1B), that terminate proliferation.

[51] http://www.ncbi.nlm.nih.gov/gene/2623

Normal stem cells, on the other hand, express master stem cell transcription factors (e.g., HLF and HOXB4) and activate stem cell genes and stem cell fate in response to the same treatments (good therapeutic index)

Namely although the CEBPA and PU.1 are high, they as transcription factors cannot function because the gene which they control has been methylated and Thus, is blocked. We demonstrate that below:



Namely with the methylation of say a promoter region even though we have a strong transcription factor it cannot function and we obtain aberrant cell growth.

11.1.1.1 HLF

We start with HLF, the hepatic leukemia factor gene and its product.

From Waters et al:

Physiological variation related to circadian rhythms and aberrant gene expression patterns are believed to modulate therapeutic efficacy, but the precise molecular determinants remain unclear. Here we examine the regulation of cell death by hepatic leukemia factor (HLF), which is an output regulator of circadian rhythms and is aberrantly expressed in human cancers, using an ectopic expression strategy in JB6 mouse epidermal cells and human keratinocytes.

Ectopic HLF expression inhibited cell death in both JB6 cells and human keratinocytes, as induced by serum-starvation, tumor necrosis factor alpha and ionizing radiation. Microarray analysis indicates that HLF regulates a complex multi-gene transcriptional program encompassing upregulation of anti-apoptotic genes, downregulation of pro-apoptotic genes, and many additional changes that are consistent with an anti-death program.

Collectively, our results demonstrate that ectopic expression of HLF, an established transcription factor that cycles with circadian rhythms, can recapitulate many features associated with circadian-dependent physiological variation.

Thus, HLF has a strong capability to allow a cell to avoid apoptosis and survive long periods. HLF is amongst the many transcription factors stabilizing cell life.

From earlier work by Honda et al, the authors had noted:

Transcription factors are frequent target of chromosomal translocations observed in acute lymphoblastic leukemia (ALL). Among them, E2A gene, encoding a basic helix-loop-helix (bHLH) transcription factor on chromosome 19, has been known to be involved in two chromosomal translocations, t(1;19)(q23;p13)3,4 and t(17;19)(p22;q13)5,6 that are observed in human B-lineage leukemias.

As a result of t(1;19)(q23;p13), the C-terminal region of E2A gene, including the bHLH DNA binding and dimerization domains, is replaced with the DNA binding domain of PBX1 homeobox gene on chromosome 1.

On the other hand, following the t(17;19)(p22;q13), the same region of the E2A gene is fused to the DNA-binding and dimerization domains of hepatic leukemic factor (HLF) gene belonging to the basic region/leucine zipper (bZIP) family on chromosome 17. These events create novel fusion gene products, E2A-PBX1 and E2A-HLF, respectively, and the expression of these chimeric transcription factors with altered structural and functional features would play a substantial role in the leukemogenic process(es).

11.1.1.2 SPI1 (PU.1)

PU.1 is another transcription factor involved in hematopoiesis. We have seen its function generally before and we have discussed its overall function. We here examine it in a bit more detail. Again, it should be noted that it is not PU.1 but the genes it transcribes that are at fault.

As Hoffman et al state (Chapter 25):

PU.1 is a member of the Ets family of transcription factors and is expressed abundantly in B cells and macrophages. Expression of PU.1 has also been reported in granulocytes and eosinophils as well as in CD34+ hematopoietic progenitor cells. Whereas high levels of PU.1 expression in fetal livers of mice preferentially direct macrophage development, low levels of PU.1 result in B-cell development. C-Jun, another member of the b-Zip family of transcription factors, serves as a coactivator of PU.1 during macrophage development.

The ETS family transcription factors form or have loops (loop-helix-loop) and are activated by kinases. (See Marks et al pp 404-406). The above continues:

It has been demonstrated that overexpression of c-Jun in myeloid progenitor cells results in macrophage development. Recent studies have revealed that downregulation of c-Jun by

C/EBPα is necessary for granulocytic maturation and appears to be the mechanism through which C/EBPα blocks macrophage development. C/EBPα not only binds to the promoter of the c-jun gene and decreases its expression but also binds PU.1, thereby inhibiting its activity.

PU.1-binding sites have been reported in almost all myeloid-specific promoters reported to date, including those for M-CSF, GM-CSF, and G-CSF receptors, all of which play critical roles in myeloid cell development. PU.1 activity is modulated both by covalent modifications and by protein–protein interactions. For example, phosphorylation of PU.1 by casein kinase II or by JNK kinase leads to increased transcriptional activity.

Abrogation of PU.1 expression in PU.1−/− mice results in perinatal lethality accompanied by the absence of mature monocytes/macrophages and B cells and delayed and reduced granulopoiesis. After in vitro differentiation, embryonic stem cells derived from PU.1−/− blastocysts fail to express mature myeloid cell markers, suggesting that PU.1 is not essential for the initial events associated with myeloid lineage commitment but is necessary for the later stages of development.

From Young et al we have a more detailed discussion on monocyte development:

Numerous studies have established that a core set of genes act as 'master regulators' of myeloid differentiation. Monocytic differentiation is promoted by the PU.1-induced transcription factors EGR2 and NAB2, whereas granulocytic differentiation is promoted by GFI1 and CEBPA .

Note the differentiation with PU.1 and CEBPA. Myeloid differentiation is complex and controlled via these transcription factors. Granulocytes result from the CEBPA lineage.

Our data show that loss of Hnrnpa0 leads to the suppression of pro-monocytic genes (Egr2, Nab2, Irf8, Emr1) and induction of pro-granulocytic genes (Cebpa, Gfi1, Csfr3), within this network. EGR1, like HNRNPA, is located within the CDS of 5q31.2, and is expressed at reduced levels in CD34+ cells from patients with a del(5q). Similar to HNRNPA0, loss of EGR1 expression favors granulocytic over monocytic differentiation.

Thus, loss of a single allele of EGR1 and HNRNPA0, as a result of a del(5q), may lead to a synergistic disruption of EGR1, and the functionally redundant family member, EGR2, leading to aberrant myeloid differentiation, a hallmark of t-MN. Any disruption to the gene program that regulates the transition from the CMP to the GMP and beyond can uncouple lineage commitment and proliferation control during myeloid differentiation and lead to malignant transformation….

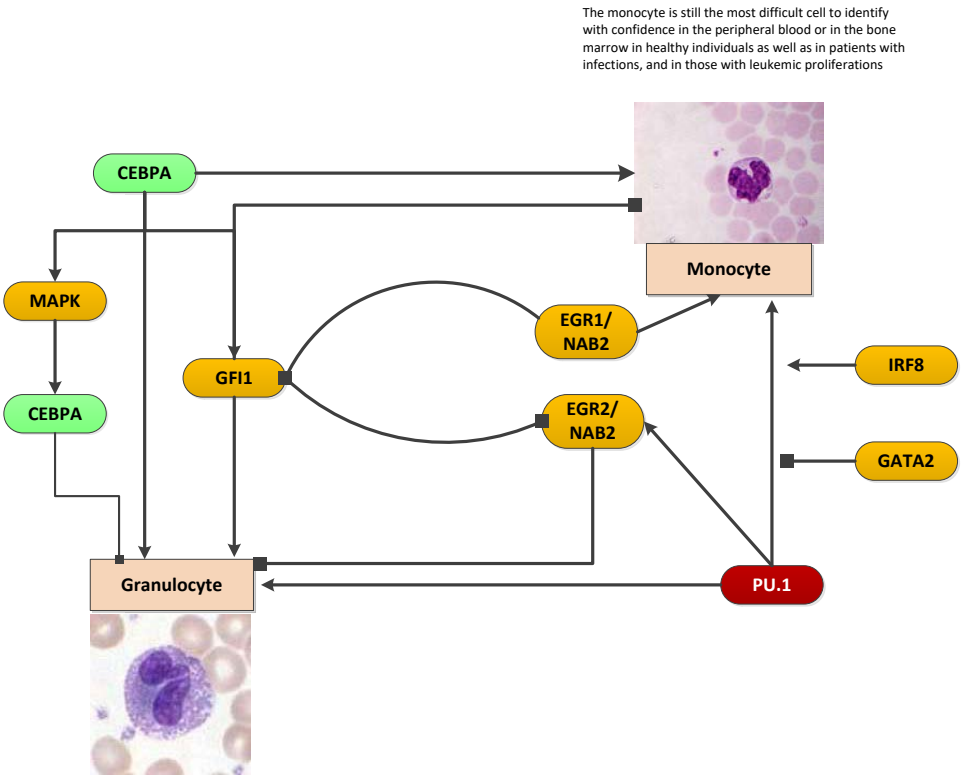
(The following Figure is as…) Model of the impact of 5q deletion upon myeloid cell fate. A schematic diagram showing some of the factors that regulate myeloid cell fate.

PU.1 normally induces EGR2 and NAB2 to induce monocytic differentiation. CEBPA and GFI1 promote granulocytic differentiation, partially through suppression of EGR1, EGR2 and NAB2.

Again, note the focus on PU.1 is monocyte and not granulocyte. Thus, when we examine a neutropenic MDS patient we are looking at a blockage of CEBPA transcription action and if the monocyte line is normal we can see a benign state there.

Knockdown of Hnrnpa0 in murine cells leads to the suppression of pro-monocytic transcripts (Egr2, Nab2, Irf8, and Emr1 (F4/80)), many of which contain AREs (light gray boxes), and induction of pro-granulocytic genes (Cebpa, Gfi1, Csf3r (G-CSFR)). MAPK-induced phosphorylation of CEBPA has been shown to inhibit granulopoeisis. KD of Hnrnpa0 leads to a decreased expression of many genes within the MAPK signaling pathway, consistent with the observed shift towards granulocytic differentiation. Loss of EGR1 (also mapped to human 5q) also favors granulocytic over monocytic differentiation36. Egr1 and Egr2 function in a redundant manner. In del(5q) patients, haploinsufficiency for both EGR1 and HNRNPA0 may synergistically disrupt myeloid differentiation.

We now demonstrate the above discussion with the Figure below. Note that we have a monocyte and a granulocyte at both ends of this process and we have included the respective transcription factors associated with each.



11.1.1.3 CEBPA

CEPBA is another key transcription factor. It is a major one especially for neutrophils and other granulocytes. As Hoffman et al state (Chapter 25):

C/EBPα (Namely CEPBA) has been postulated to be a master regulator of the granulocytic developmental program. It is expressed at high levels throughout myeloid differentiation and has been shown to bind to the promoters of multiple myeloid-specific gene promoters regulating gene expression at many different stages of myeloid maturation. Although C/EBPα−/− mice die perinatally because of defects in gluconeogenesis that result in fatal hypoglycemia, they also have a selective early block in the differentiation of granulocytes without affecting either monocyte/macrophage maturation or the differentiation of other hematopoietic lineages. Myeloid cells from C/EBPα−/− mice lack the G-CSFR, and it has been postulated that lack of mature neutrophils in these mice may be caused by the lack of G-CSFR. However, the myeloid defect in C/EBPα−/− mice is more severe than that seen in G-CSFR−/− mice, suggesting that C/EBPα has additional functions vital to granulocytic maturation.

C/EBPα is a single exon gene, but it is expressed as two isoforms that arise from alternate translation start sites that give rise to a full-length C/EBPαp42 and a truncated dominant negative C/EBPαp30 isoform.13 Translational control of C/EBPα isoform expression is orchestrated by a conserved upstream open reading frame (uORF) in the 5' untranslated region (5'UTR). This region is thought to be responsive to the activities of the translation initiation factors eIF4E and eIF214 such that an increase of eIF2 or eIF4E activity results in an increase in expression of the shorter p30 isoform.13

Again, it appears that CEBPA is expressed but that when it is so the transcription site is blocked due putatively to methylation From Pabst and Mueller:

This review intends to highlight recent reports on dysregulation of the differentiation factor CEBPA at various levels in human AML. The CCAAT enhancer binding protein alpha (CEBPA) is a member of the basic region leucine zipper family of transcription factors. It is composed of two transactivation domains in the N-terminal part, and a leucine zipper region mediating dimerization with other CEBP family members and a DNA binding domain in the C-terminal part.

As a condition for DNA binding, dimerization depends on the basic amino acid residues, and genomic alterations in the exact distance between basic region and leucine zipper impair DNA binding. Two inframe start codons give rise to two CEBPA isoforms: The p30 protein is initiated at an AUG codon further downstream and Thus, lacks the amino terminal sequences, whereas the C terminus is identical to the full-length p42 protein.

As a consequence, the p30 isoform lacks domains mediating the contact with the transcriptional apparatus, whereas other functions such as dimerization or regions involved in protein-protein interactions are preserved in both p30 and p42 proteins. CEBPA is an intronless gene located at chromosome 19q13.1. It was originally isolated as a rat liver transcription factor regulating hepatic and adipocyte genes. Studies in adipocyte lines have founded the role of CEBPA as an inhibitor of cell proliferation and as a tumor suppressor. In hematopoiesis, the interest in CEBPA is based on its crucial role during the development of granulocytes and on its deregulation associated with myeloid transformation

Isoforms are presentations which may alter the effectiveness of CEBPA.

As Sonnet et al state:

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy associated with severe morbidity and poor prognosis. It comprises a highly heterogeneous group of blastic myeloid malignancies and constitutes the most frequent type of acute leukemia in adults [1]. AML can arise de novo but also secondarily from preceding myelodysplastic syndrome (MDS), or after cytotoxic treatment or radiotherapy. It is characterized by an aggressive clonal proliferation of immature hematopoietic progenitor cells (myeloblasts) and impaired differentiation.

Recurrent chromosomal aberrations and rearrangements occur in more than 50% of cases and represent important predictive factors for response to therapy and outcome of the disease.

Altered gene function in AML is often a consequence of distinct cytogenetic aberrations, but also results from mutations in genes like CEBPA (CCAAT/enhancer-binding protein, alpha), FLT3 (fms-like tyrosine kinase receptor-3), or NPM1 (nucleophosmin 1. Although novel high resolution genome-wide technologies have enabled the detection of numerous gene mutations, the multistep process of leukemogenesis is still poorly understood.

It is not the mutation of CEBPA in MDS that is the issue but its failure to transcribe. However, it appears that in progression to AML that CEBPA itself is mutated.

11.1.1.4 GATA1

We now consider GATA1 and its impact. We summarize some details but the concern as we shall show is that GATA1 control an alternative line. Let us begin with a summary of GATA1 operations.

From Morceau et al:

The zinc finger protein GATA-1 is considered as one of the most critical transcription factors in erythropoiesis as well as megakaryopoiesis. Besides GATA-1 that belongs to the GATA-family of transcription factors, GATA-2 is also involved in erythropoiesis and megakaryopoiesis regulation.

Both GATA-1 and GATA-2 transactivation activities require interaction with friend of GATA (FOG)-1 cofactor. In addition, both transcription factors have GATA binding sites in their cis-acting elements allowing a cross-regulatory mechanism in which GATA-1 can control the expression of GATA-2 and vice versa. GATA-2 is overexpressed in early immature hematopoietic progenitors to ensure their maintenance and proliferation whereas GATA-1 is essential for the survival of erythroid progenitors as well as the terminal differentiation of erythroid cells. In fact, increased expression of GATA-2 determines megakaryocytic differentiation whereas its down-regulation is required for erythroid differentiation.

Recently, a role for GATA-2 in the regulation of quiescence in human hematopoietic stem and progenitor cells has been reported. GATA-1 activation has been correlated to its phophorylation. Epo-induced phosphorylation of GATA-1 is important for maturation of fetal liver erythroid progenitor cells, specifically on serine 310 by PI3K/AKT that enhances GATA-1 transcriptional activity in vitro and in erythroid cells.

However, GATA-1 acetylation by CBP/p300 is also described as crucial for the binding to its DNA target GATA sequence possibly involving phosphorylation. Moreover, phosphorylation of GATA-1 could be mediated by MAPK pathway, as a ubiquitination signal for its proteasomal degradation.

On the other hand, besides FOG1, GATA-1 activity is highly dependent on interaction with many cofactors including EKLF, SP1, CBP/p300, Lmo2, Ldb1, RUNX1, Fli1 and PU.1, which represent a part of the best-described interacting proteins. These cofactors can constitute a very complex network regulating erythropoiesis and megakaryopoiesis, by promoting or repressing GATA-1 activity.

Particularly, PU.1 is a strong inhibitor of GATA-1 DNA-binding activity and erythroid differentiation.

Before examining this observation, it is worth a brief review of the NF-kB gene complex. We demonstrate this below (taken from Marks et al pp 408-416). The Figure below is a modification from Marks et al of the NF-kB complex. It functions as follows:

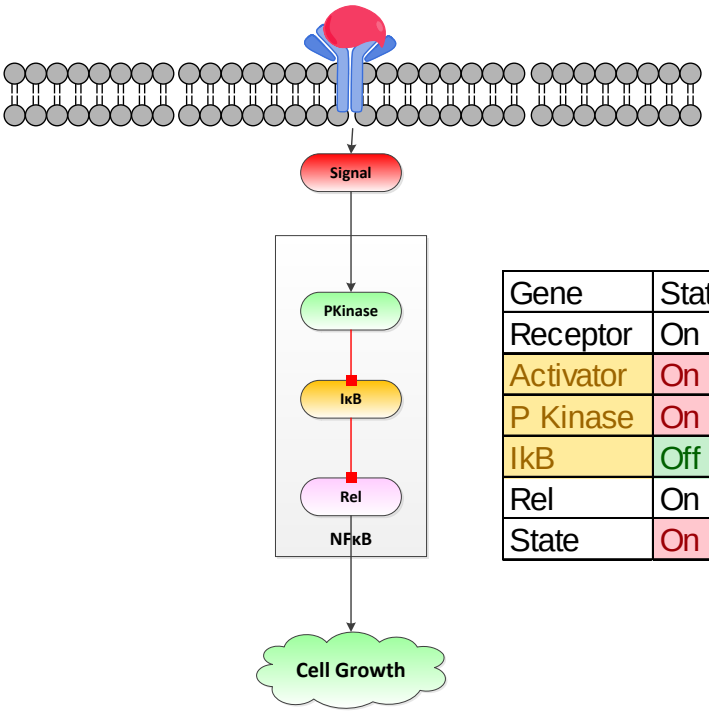
1. A receptor is activated via a ligand starting a path down a signalling chain to the NF-kB complex.

2. This signal then activates the PK kinase which can block the IkB portion of the transcription factor.

3. The transcription factor is the combined IkB and Rel proteins.

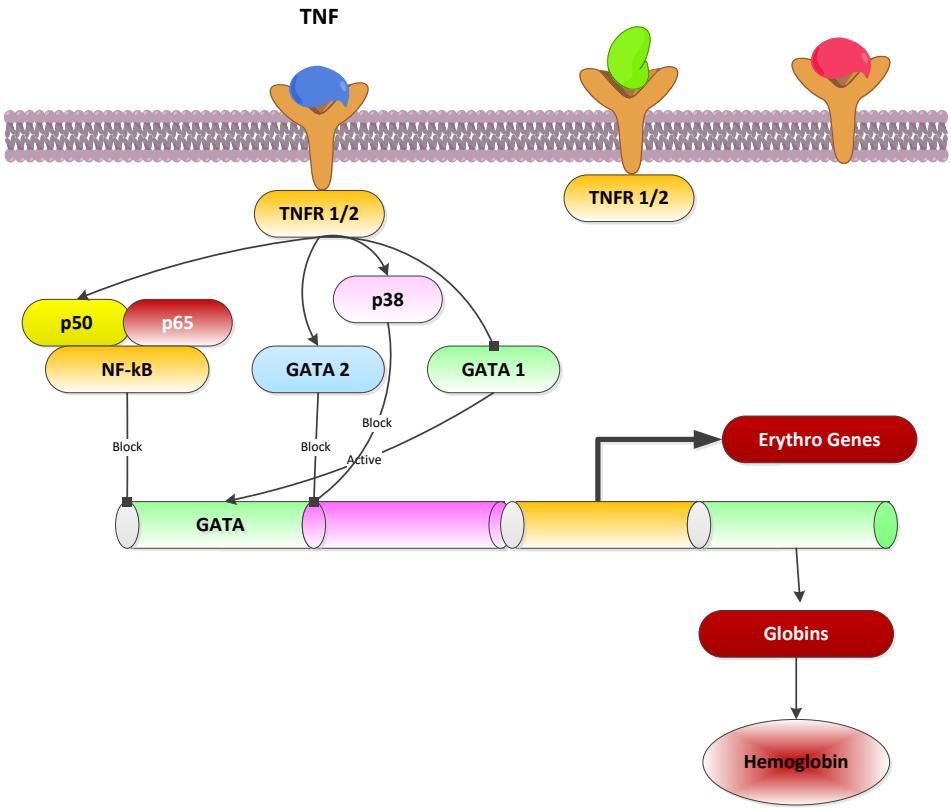
4. The Rel protein, if allowed to be activated, can facilitate survival and development.

5. Note that there is a switch mechanism in the NF-kB complex. If it is activated, then PK blocks IkB and no IkB means Rel is active and Thus, we have the result. If on the other hand there is no input activation then PK is not active and IkB is not blocked but active, and Thus, Rel is blocked, and there is no survival or development resulting from Rel transcription.
6.



Gene	State	State
Receptor	On	Off
Activator	On	Off
P Kinase	On	Off
IkB	Off	On
Rel	On	Off
State	On	Off

Now using the above from Morceau et al we have the following Figure demonstrating GATA1 and its activation.

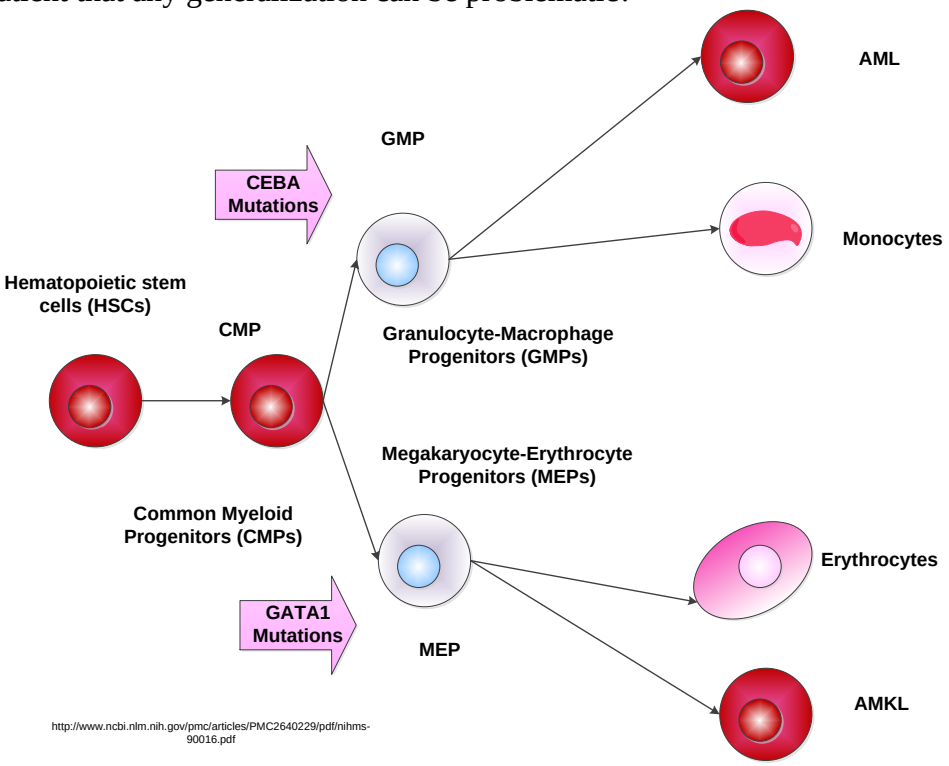


However, with GATA1 we really have control over an alternative line and Thus, there is a concern as to what GATA1 deviation truly measures. From Look:

Mutations in the gene encoding the transcription factor GATA1 contribute to a specific type of leukemia in people with Down syndrome. This finding suggests a multistep pathway of leukemia development that involves GATA1 and one or more genes on chromosome 21.

Human myeloid leukemias are clonal disorders arising from the accumulation of mutations in hematopoietic stem cells (HSCs) that retain the capacity for self-renewal1. Most leukemogenic mutations are acquired somatically and affect a growing cadre of proto-oncogenes and tumor suppressor genes.

From Look we have the following Figure discussing this issue as stated above. Note that we have two arms of progression. The CEBA line leads to an AML path whereas the GATA1 line leads to an AMKL path with erythrocytes. Both are myeloid products but they grow into two separate lines; erythroid and monocytes. For example, neither is neutrophil linked. Thus, one may ask if such a study on MDS with such a disparate set of expressions when agglomerated makes any sense in separating factors. Specifically, we may reasonably ask; is MDS so different from patient to patient that any generalization can be problematic?



We now move to several observations which can be made.

Also from Hoffman et al there is a detailed discussion regarding GATA1 and AMKL[52]:

[52] Novel Somatic Mutations in JMML, Dr. Carl Allen, MD, PhD; Baylor College of Medicine, Date Published: 10Mar2014, On Line in Hoffman et al. Chpt 62.

The molecular pathogenesis of TMD and AMKL in children with Down syndrome is now providing valuable insights into myeloid leukemogenesis.20 Recent studies have shown that virtually all patients with TMD and most patients with AMKL harbor mutations in the hematopoietic transcription factor GATA1.

GATA1 is a double zinc finger DNA-binding transcription factor expressed primarily in hematopoietic cells. It is required for the development of red blood cells, megakaryocytes, mast cells, and eosinophils. A number of different mutations in GATA1 have been identified, including insertions, deletions, missense mutations, nonsense mutations, and slice site mutations. All of these mutations lead to a block in the expression of the full-length 50-kd isoform of GATA1 but allow for the expression of a smaller, 40-kd isoform (GATA1s).

The zinc finger transcription proteins have loops of nucleic acids bound by two zinc molecules and then the bottom loop portions can bind as a transcription factor to the DNA and assist in effecting transcription. Mutations are possible but it is essential to still have the exposed loops between each of the zinc fingers.

This smaller isoform lacks the N-terminal transactivation domain but retains both zinc fingers involved in DNA binding as well as interactions with its cofactor, friend of GATA1 (FOG1). Recent studies have shown that mutations that alter GATA1–FOG1 binding in the N-terminal zinc finger or result in the expression of the GATA1s isoform uncouple megakaryocyte growth and differentiation.

Similar studies in cell lines derived from children with Down syndrome and AMKL have demonstrated that expression of GATA1 led to erythroid differentiation whereas expression of GATA1s did not alter the characteristics of the cell line. Taken together, current data suggest that the loss of GATA1 and expression of GATA1s directly contribute to leukemogenesis.

It Thus, appears that GATA1 is a crucial gene. However, in the article in question the GATA1 seem to still be present but that their ability to effect a transcription factor is blocked by methylation.

Although mutations in GATA1 may be sufficient to cause TMD, these mutations are not sufficient for the development of AMKL, as evidenced by the latency period between resolution of TMD and the development of AMKL as well as the observation that not all children with TMD and GATA1 mutations will ultimately develop AMKL. Therefore, a multistep pathogenesis model is proposed in patients with Down syndrome in whom AMKL develops in clones with GATA1 mutations and additional cooperating mutations.

One potential "second-hit" mutation may occur in the JAK3 (Janus kinase 3) gene, a member of the JAK family of nonreceptor tyrosine kinases. Several gain-of-function mutations and loss-of-function mutations in JAK3160160 have been identified in both TMD and AMKL patient samples. Although mutations in JAK3 might indeed represent a "second hit," the finding of mutations in TMD patients who have not progressed to AMKL may argue against JAK3 as a second cooperating mutation.

Thus, examining the effect of loss of function in genetically changed patients is helpful in understanding its function overall.

Epigenetic factors are appearing to be more prevalent in our understanding of the causes of many cancers. These factors include such elements as methylation, long non-coding RNAs (lncRNA), micro RNAs and acetylation. None of these reflect a fundamental change in the DNA of the underlying genes, but they do reflect a complex process whereby the way the DNA is processed and presented functions. Unlike translocations and gene changes which are difficult to unravel, many of these epigenetic changes may be found to be reversible in part or in whole. We focus on methylation and methylation related disorders herein.

11.1.2 The MDS Therapeutic Paradigm

MDS, the myelodysplastic syndrome, is a multifaceted disease of the bone marrow cells which leads to the over-production of immature blood cells; erythrocytes, lymphocytes, platelets and others. It is often indolent in its early stages but then turns quite virulent and is often fatal, frequently due to the development of AML, acute myelogenous leukemia. However, recent understanding of a key driver of MDS, namely hypermethylation, has resulted in complex therapies which may have proven not only efficacious but curative.

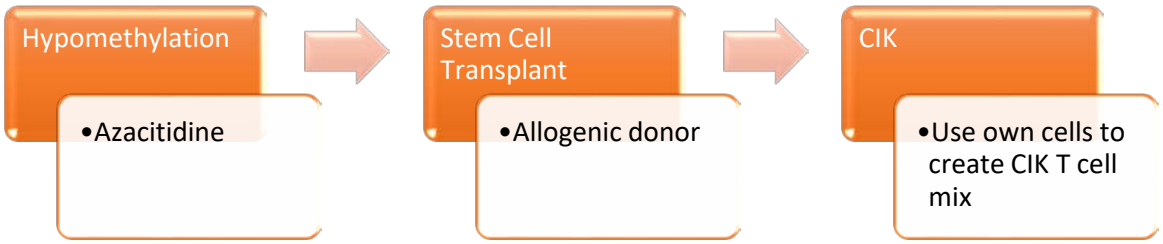
We use this disorder as an example of how methylation causes potential cancers and further how it can be targeted and treated.

The therapeutic responses to MDS are representative to the multi-prong attack on various cancers. The fact that MDS is not per se a cancer but an artifact of a hypermethylation state, and that hypermethylation can be reversed, as compared to a genetic change such as found in CML, the Philadelphia chromosome translocation, and that we know how to deal with hypermethylation, lends MDS to some form of initial treatment. However, demethylation does not always work.

Thus, the second attack is more aggressive which is a modified hematologic stem cell transplant.

That further reduces the aberrant cell load to an almost miniscule amount. The final hit is using modified T cells called cytokine induced killer cells specifically targeted for the remaining

hypermethylated cells. We depict this below:



This paradigm has been applied to other malignancies with substantial success. The classic cases are the childhood leukemias and Hodgkin's lymphoma. One would suspect that MDS being substantially of the same class would fit this paradigm. Our intent here is to examine the literature across the above spectrum and attempt to make an assessment of progress in this disease.

11.1.3 Historical Context

Methylation has been known of for decades but it has only been in the last fifteen years or so that the connection between methylation and cancers has been somewhat understood. In a 1997 paper by Jones and Gonzalgo the authors state:

DNA methylation is a mechanism for changing the base sequence of DNA without altering its coding function. As a heritable, yet reversible, epigenetic change, it has the potential of altering gene expression and has profound developmental and genetic consequences. The methylation reaction itself is mechanistically complex and involves the flipping of the target cytosine out of the intact double helix, so that the transfer of the methyl group from S-adenosylmethionine can occur in a cleft in the enzyme.

Cytosine methylation is inherently mutagenic, which presumably has led to the 80% suppression of the CpG methyl acceptor site in eukaryotic organisms, which methylate their genomes. It contributes strongly to the generation of polymorphisms and germ-line mutations, and to transition mutations that inactivate tumor-suppressor genes. Despite a 10- to 40-fold increases in the rate of transitions.

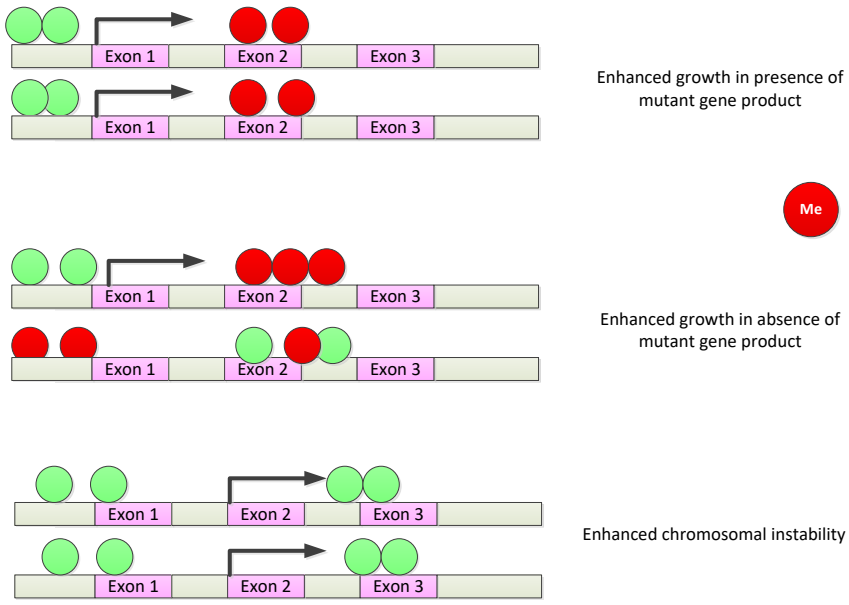
This was somewhat of an opening salvo regarding methylation and cancers. One should remember that this was almost five years before the complete reading of human DNA and also at a time when actually reading the methylated states was complex at best.

The authors hypothesized a mechanism for uncontrolled growth using the methylation construct. They posited three ways in which methylation functioned.

First, it caused a gene change. This was the C to T mutation change.

Second, they posited the promoter suppression via methylation of the promoter. This method is seen quite frequently in the process.

Third, there may be a chromosome instability resulting from methylation.



At the same time, Robertson and Jones wrote a paper on DNA methylation and its affects and they also suggested a strong link between that and cancer. They stated:

As with the demethylation and de novo methylation observed during development, changes in methylation patterns during neoplasia have been recognized for some time. Initially it was shown that malignant cells have lower levels of methylation than do normal cells. This global hypomethylation accompanies a hypermethylation of CpG islands, DNA regions often associated with promoters of human genes that are normally protected from methylation.

The above statement is a clear description of what we now know to be correct; namely hypomethylation globally but hypermethylation of the CpG islands. The hypomethylation allows expression of a wide variety of proliferation genes while the CpG Island silencing via hypermethylation deactivates control genes. They continue:

The mechanism by which these regions remain unmethylated in the normal cell is not known, but it may be mediated by the binding of certain transcription factors. In malignant cells, these CpG-island regions become methylated and expression of the associated gene is silenced. In the case of a tumor-suppressor gene, this may result in a growth advantage for the cell.

DNA methylation– mediated transcriptional inhibition has Thus, been proposed as a mechanism that is alternative to mutation and deletion, in the removal of tumor suppressor– gene function. Examples of such genes include the two cell-cycle regulators p16 Ink4a and p15 Ink4b, the von Hippel–Lindau gene VHL in some renal carcinomas, the retinoblastoma gene product Rb, BRCA1, the angiogenesis inhibitor thrombospondin, and the metastasis-suppressor gene E-cadherin.

… Chuang et al. have shed new light on how methylation patterns are maintained and how they may of the associated gene is silenced. In the case of a tumor-suppressor gene, this may result in a growth advantage for the cell. DNA methylation– mediated transcriptional inhibition has Thus, been proposed as a mechanism that is alternative to mutation and deletion, in the removal of tumor suppressor– gene function.

Examples of such genes include the two cell-cycle regulators p16 Ink4a and p15 Ink4b, the von Hippel–Lindau gene VHL in some renal carcinomas, the retinoblastoma gene product Rb, BRCA1, the angiogenesis inhibitor thrombospondin, and the metastasis-suppressor gene E-cadherin (Graff et al. 1995). In a recent issue of Science ...has shed new light on how methylation patterns are maintained and how they may become altered in cancer. It was shown that the DNA methyltransferase is targeted to newly replicated DNA by the replication associated protein PCNA (proliferating cell nuclear antigen).

PCNA is the polymerase-processivity factor for the d and e DNA polymerases, is homologous to the E. coli b subunit, and is required for DNA replication becomes altered in cancer. It was shown that the DNA methyltransferase is targeted to newly replicated DNA by the replication associated protein PCNA (proliferating cell nuclear antigen). PCNA is the polymerase-processivity factor for the d and e DNA polymerases, is homologous to the E. coli b subunit, and is required for DNA replication

11.1.4 Why Study MDS?

There are slightly more than 10,000 new cases of MDS each year. There may be a little difficulty in determine them because they can often go un-noticed until they convert to AML at which point the diagnosis would be clear. There may be a slight anemic, thrombocytopenia, and the presence of blasts, immature hematopoietic cells. A true diagnosis requires a bone marrow biopsy. The MDS patient may have one of many variants which we shall discuss latter.

However, what seems common is the presence of hypermethylation resulting in the suppression of cell growth and proliferation control genes on the lineage of hematopoietic cells first affected. Thus, the thrombocytes may be the initial ones affected and we see a drop-in platelet and a presence of blasts. But in all cases, it is the hypermethylation. There is as of yet in the process no genetic change, the excess immature growth is due solely to hypermethylation. Thus, the control

is simply control of hypermethylation via drugs which block the process. It is a somewhat simple model for developing a therapeutic.

Thus, why study MDS? The answers are:

1. MDS is not a full-blown cancer. It lacks the genetic breakdown.

2. MDS is a hypermethylation disease. Hypermethylation can be reversed. Thus, there is an opportunity to seek a "cure".

3. MDS does lead to cancer, most likely AML. The process that results in that change is worth of study as a means to seek both prevention and cure.

4. MDS can be monitored both genetically as well as via hypermethylation measurements.

11.2 Overview

In this report, we examine several factors in depth. Specifically:

MDS: We present an overview of MDS and its various forms. This is a complex disease and it is almost as if no one patient is identical to any other patient. We consider the cause of methylation at the DNA level but we can at best speculate on the ultimate initiator. We know that many MDS patient had pre-existing malignancies for which the received both chemotherapy and radiation therapy. The nexus there seems to somewhat clear. However, many, if not most, MDS patients have no clearly defined initiating event.

Methylation: We explore methylation and examine how it occurs, and what it does to the functioning of the DNA expression. In many of our cancer models we often just look at gene, RNA and protein flow. As we have indicated before we often look at the epigenetic factors as noise. However, it has become clear that the epigenetic elements are integral parts of a cells expression of its genetic capabilities and Thus, should be included in any model.

Demethylating Therapies: We examine the various demethylating therapies. The specifics are discussed in some detail as well as the efficacy of the therapeutics.

Acetylation: The histones around which the DNA is wound also exhibit acetylation. We examine this phenomenon and relate it to methylation.

Immunotherapy: We discuss immunotherapy focusing on the use of CIKs, cytokine induced killer cells, primed T cells directed at the remaining methylated hematopoietic cells.

We conclude with observations relevant to combined therapies.

11.2.1 MDS

We will now examine MDS from a clinical perspective. MDS is a complex set of disorders. There is no single measurement and almost every patient a physician may see presents a somewhat unique set of abnormalities. At the heart of all is a cytopenias of the blood, anemia, thrombocytopenia, leukopenia, and the like. In some cases, as is often the case, it is found as an incidental finding on a blood test leading to more detailed bone marrow studies.

To define MDS we use the recent work of Tefferi and Vardiman who state:

The main feature of myeloid neoplasms is stem-cell–derived clonal myelopoiesis with altered proliferation and differentiation. The phenotypic diversity of these neoplasms has been ascribed to different patterns of dysregulated signal transduction caused by transforming mutations that affect the hematopoietic stem cell. There is increasing evidence that haploinsufficiency, epigenetic changes, and abnormalities in cytokines, the immune system, and bone marrow stroma all contribute to the development of the myelodysplastic syndromes.

The WHO Classification of MDS is as in the following:

1. Acute myeloid leukemia and related neoplasms*
2. Myelodysplastic syndromes
    a. Refractory cytopenia with unilineage dysplasia†
        i. Refractory anemia (ring sideroblasts <15% of erythroid precursors)
        ii. Refractory neutropenia
        iii. Refractory thrombocytopenia
    b. Refractory anemia with ring sideroblasts (dysplasia limited to erythroid lineage and ring sideroblasts ≥15% of bone marrow erythroid precursors)
    c. Refractory cytopenia with multilineage dysplasia (regardless of ring sideroblast count)
    d. Refractory anemia with excess of blasts (RAEB)
        i. RAEB-1 (2–4% circulating blasts or 5–9% marrow blasts)
        ii. RAEB-2 (5–19% circulating blasts or 10–19% marrow blasts or Auer rods present)
    e. Myelodysplastic syndrome with isolated del(5q)
    f. Myelodysplastic syndrome (unclassifiable)
3. Myeloproliferative neoplasms
4. Myelodysplastic–myeloproliferative neoplasms
5. Molecularly characterized myeloid or lymphoid neoplasms associated with eosinophilia

The presence of excess blasts is generally the telling factor. Yet as the above authors state:

The minimal morphologic criterion for the diagnosis of a myelodysplastic syndrome is dysplasia in at least 10% of cells of any one of the myeloid lineages. However, such changes can also be seen in other myeloid neoplasms, which must be excluded before a diagnosis is made. These include AML, which is defined by at least 20% myeloblasts in bone marrow or peripheral blood; MDS– MPN, in which dyserythropoiesis or dysgranulopoiesis is associated with leukocytosis or monocytosis (>1.0×109 cells per liter), as in CMML; and MPN, in which both dyserythropoiesis and dysgranulopoiesis are absent.

From Greenberg et al we have the following IPSS characterization. First the cytogenetic abnormalities must be evaluated. They fall into 5 categories as shown below. One can have from 1 to over 3 abnormalities and the greater the number the higher the risk of low survival.

Prognostic subgroups (% patients)	Cytogenetic abnormalities	Survival* Years, median	AML evolution, 25%* Years, median	Hazard ratios OS/AML*	Hazard ratios OS/AML^
Very good (4%*/3%^)	-Y, del(11q)	5.4	NR	0.7/0.4	0.5/0.5
Good (72%*/66%^)	Normal, del(5q), del(12p), del(20q), double including del(5q)	4.8	9.4	1/1	1/1
Intermediate (13%*/19%^)	del(7q), +8, +19, i(17q), any other single or double independent clones	2.7	2.5	1.5/1.8	1.6/2.2
Poor (4%*/5%^)	-7, inv(3)/t(3q)/del(3q), double including -7/del(7q), complex: 3 abnormalities	1.5	1.7	2.3/2.3	2.6/3.4
Very poor (7%*/7%^)	Complex: >3 abnormalities	0.7	0.7	3.8/3.6	4.2/4.9

Then we take the complete set of five measurements and assign them to the following Table and create a score based upon each one, the result being the cumulative score.

Prognostic variable	*0*	*0.5*	*1*	*1.5*	*2*	*3*	*4*
Cytogenetics	Very Good		Good		Inter-mediate	Poor	Very Poor
BM Blast %	2		2%-5%		5-10%	>10%	
Hemoglobin	10		8-<10	<8			
Platelets	>100,000	50,000-100,000	<50,000				
ANC[53]	0.8	<0.8					

As above the cytogenetics can be 0, or 0.5 to 4.0. The blast score is 0, 1 or 1.5. The platelets can be 0, 0.5 or 1.0. Finally, the ANC, absolute neutrophil count, may be high or low, and Thus, 0 or 0.5.

[53] See: http://www.mdanderson.org/patient-and-cancer-information/cancer-information/cancer-types/myelodysplastic-syndrome/index.html Absolute Neutrophil Count (ANC) is a measure of the number of WBCs you have to fight infections. You can figure out your ANC by multiplying the total number of WBCs by the percentage of neutrophils ("neuts"). The K in the report means thousands. For example:

WBC = 1000 = 1.0K
Neuts = 50% (0.5)
1000 X 0.5 = 500 neutrophils

Category	*Score*
Very Low	≤1.5
Low	1.5-3.0
Intermediate	3.0-4.5
High	4.5-6.0
Very High	>6.0

Thus, we have a patient with the following profile:

1. 1 cytogenetic abnormality; they score Good and have a value 1.0
2. blasts in excess of 15% that yields a 3.0
3. Hemoglobin of 12: that is 0.0
4. Platelets at 80,000 that is a 0.5.
5. ANC in excess of 0.8 that is 0.0

The total score is 4.5. This patient is borderline on Intermediate and High Risk.

11.2.2 Epidemiology and Etiology

Let us examine the overall epidemiology of MDS. From DeVita et al Chapter 135 (8th Ed) we have:

The incidence of MDS in the United States is reported to be 3.4 per 100,000 persons.4 MDS is rare in patients younger than 50 years, but can reach as high as 20 to 50 per 100,000 in individuals older than 70 years.

With around 15,000 new patients diagnosed every year in the United States, MDS has become one of the most common disorders in the section of leukemias. The increase in incidence that is currently observed may relate to increased reporting by clinicians and pathologists. Reasons for not diagnosing MDS in the past may have included little interest in pursuing this diagnosis, especially in older patients (perceived lack of effective therapy other than supportive, comorbidities), and overlap of MDS with other disorders (aplastic anemia, myeloproliferative diseases, and AML).

Thus, the incidence is quite small, as say compared to prostate or breast cancer and the complexity of the cellular presentation is also quite complex. After examining the putative predisposing causes we can find that given further the condition of the patient that each case is almost unique, unlike some of the more common cancers.

Let us now continue with etiology.

No etiologic factor is identified in most patients with MDS. MDS is more frequent in men than women by a factor of 1.8.4 It has been associated with smoking and hair dyes, exposure to agricultural and industrial toxins, drugs (e.g., chloramphenicol), and occupational exposures to stone and cereal dusts. MDS has been associated with exposure to ionizing radiation (atomic

bomb survivors in Japan, decontamination workers following the Chernobyl nuclear plant accident) and chronic exposure to low-dose radiation (radiopharmaceuticals).5,6 Some inherited hematologic disorders (Fanconi anemia, dy-skeratosis congenita, Shwachman-Diamond syndrome, Diamond- Blackfan syndrome) are also associated with a higher risk of MDS.

There is the putative cause due to such chemicals as benzene and others but this is really limited evidence.

About 20% to 30% of patients with MDS have therapy-related MDS (t-MDS).7 Distinct clinical features have been described based on the nature of the triggering event. t-MDS following exposure to alkylating agents has a longer latency period (3 to 8 years) and is often associated with abnormalities of chromosomes 5 and 7; the latency period following topoisomerase II inhibitors is shorter (2 to 3 years), and cytogenetic- molecular abnormalities tend to involve rearrangements of the MLL gene on chromosome 11q23. Risk factors associated with t-MDS include the cumulative dose of alkylating agents (e.g., cyclophosphamide, melphalan, procarbazine, chlorambucil) or topoisomerase II inhibitors (e.g., etoposide), previous radiation exposure, older age, and use of radiotherapy prior to transplantation.

Indeed, a significant number of patients have a clear path backward with aggressive radiation treatment. This is not just casual radiation but extensive radiation.

The number of patients with t-MDS is increasing because of better outcome for tumors that formerly lacked effective therapy. The incidence of t-MDS following therapy for other hematologic (e.g., Hodgkin's disease, non–Hodgkin's lymphoma, chronic lymphocytic leukemia) or non-hematologic malignancies (e.g., breast or testicular cancers) is between 1% and 15% according to which particular study and malignancy is concerned.8–12 Secondary and t-MDS are distinguishable from primary MDS by an earlier age of onset, more prominent dysplasia, more severe cytopenias, more rapid progression to AML, and worse outcome. The worse prognosis may be related to a higher frequency of poor-prognosis cytogenetic abnormalities in these cases.

The causes are often uncertain. As Stanford Medical Center states[54]:

People who have received radiation therapy, chemotherapy with alkylating agents (such as chlorambucil, cyclophosphamide, and melphalan), or who have been exposed to industrial solvents (such as benzene) have a higher risk of developing MDS than people who have not had these exposures. Rarely, genetic disorders are responsible for the disease. Nevertheless, in 60% to 70% of MDS patients, no specific cause can be identified.

Thus, women who have been treated for breast cancer may very well have been pre-conditioned or those in the chemical field who may have dealt with benzene.

[54] http://cancer.stanford.edu/blood/mds.html

11.2.3 Methylation

Methylation is a recently understood process and is a part of the overall set of epigenetic factors which control the classic Watson-Crick paradigm. In classic Watson-Crick structures we have genes (DNA) to RNA to proteins. The next step is the feedback mechanisms amongst proteins and genes. The next step is the collection of dogs and cats we call epigenetic factors. In this section, we present an overview of methylation, one of the epigenetic factors and one which dominates in MDS as well as many other cancers.

As Issa and Katarjian state:

We all start life thanks to inhibition of DNA methylation. As soon as embryogenesis begins, a massive decrease in DNA methylation reprograms the epigenome and creates a nearly blank slate on which development and differentiation can be written. Thus, a decrease in DNA methylation is compatible with life, at least in embryogenesis. Nuclear transplantation-induced reprogramming can also erase (if incompletely) DNA methylation in adult cells and, when applied to cancer, seems to reverse the malignant phenotype, even in the face of genetic alterations.

Outside of epigenetic reprogramming, inhibition of DNA methylation can only be achieved by genetic or pharmacologic targeting of DNA methyltransferase enzymes. Given that DNA methylation is a post-DNA synthesis event that needs to be sustained by the presence of methylating enzymes, cellular replication in the face of reduced levels of these enzymes results in significant demethylation in daughter cells, accompanied by gene reactivation.

When applied to cancer cells, this approach does have a therapeutic ratio; normal cells tend to survive hypomethylation whereas cancer cells tend to be killed (or at least stop proliferating) when this happens, perhaps because cancer cells are dependent on critical gene silencing for survival (whereas normal cells are not).

There is a possible restatement of the last paragraph. Some cancer cells if hypomethylated proliferated because the proliferation genes are not deactivated. Thus, hyper methylation is not necessarily related to cancer and hypomethylation not so.

11.3 BASIC PRINCIPLES

DNA methylation is a process whereby the cytosine is changed by the insertion of a methyl group on the 5 carbon of the ring. It is a process which is epigenetic and can dramatically modify gene expression. In fact, many of the methylation issue in humans are also common to plants, see the work by Zilberman. There has been a great deal of work demonstrating the impact of methylation on cancer progression; specifically, the recent summary by Herman and Baylin, that of Palii and Robertson, that of Robertson and Wolffe, Strathdee and Brown, Calin and Croce, are all worth reviewing.

In this report, we examine methylation and its impact on several cancers. We will also examine briefly the causes of methylation as well as the therapeutics in use to modulate cancers that cause or persistence is supported by methylation related products, either directly or indirectly.

In the paper by Das and Singal, the authors define epigenetics in a quite clear manner:

Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence.

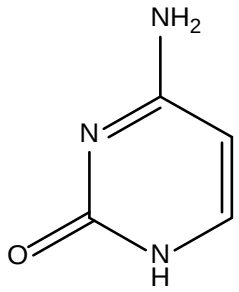
DNA methylation is one of the most commonly occurring epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target.

Epigenetics has evolved as a rapidly developing area of research.

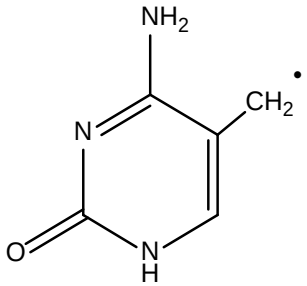
Recent studies have shown that epigenetics plays an important role in cancer biology, viral infections, activity of mobile elements, somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation

This is one of the clearest definitions of epigenetics and especially the linking of methylation to epigenetics. The classic Watson and Crick model, now some 60 years old, we had the paradigm of DNA, RNA and protein. It was the proteins which did the work. In the 1953 world, the proteins stood one by one and the clarity of gene to protein was unquestioned. Yet as we have come to better understand the details, and the details always count, there are many interfering epigenetic factors that all too often get in the way. Methylation is but one of those factors.

Basic cytosine is shown below. It has two NH groups at opposite poles and single oxygen.



Now when the 5 carbon is replaced by a methyl group we obtain the form below. This is methylated cytosine.



Thus, this small change in C, by adding the methyl group, can make for a dramatic difference in the expression of genes. For example, a well-controlled gene for proliferation, such as PTEN, may have its control over-ridden by the methylation of Introns of CpG islands, namely collections of C, cytosine nucleotides, and G, guanine nucleotides. The introns may be down from the gene, they may even be on a promoter section. The impact could aberrant cell proliferation and growth.

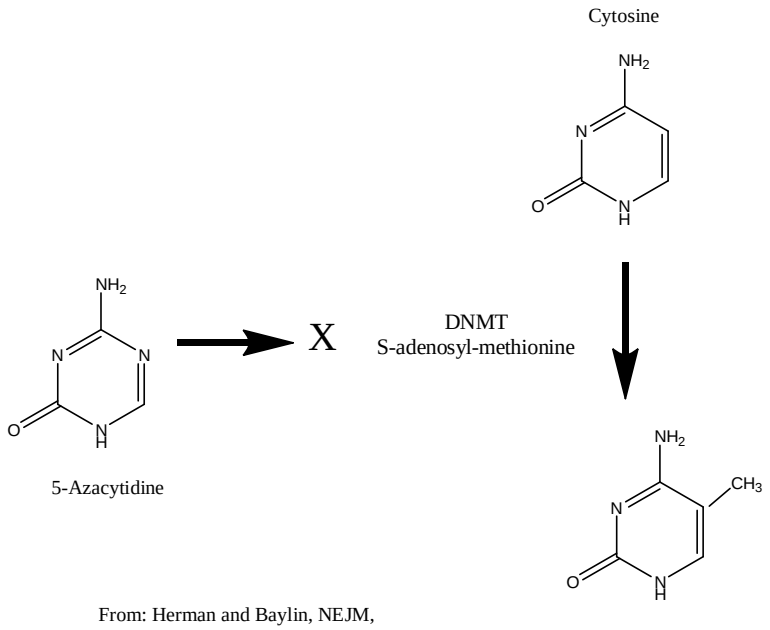
We examine the process; we then look at three types of cancers, a glandular, an epidermal, and a hematopoietic form and then examine some means used to control those cancers through the understanding or methylation and the control of it by therapeutics designed just for that purpose.

What is important about understanding methylation and especially all epigenetic changes are that it may perhaps be simpler to control them rather than a gene mutation. As Brower states:

The move from a purely genetic to an epigenetic model is crucial for prevention strategies. As numerous gene therapy trials have shown, it is very difficult to treat a genetic disease by re-activating the dormant, mutated gene or by replacing it with a non-mutated one. "Epigenetic changes are reversible, and therefore have an edge over genetics," says Mukesh Verma, an epigeneticist at the National Cancer Institute's division of cancer control and population sciences in Bethesda, Maryland. Furthermore, epigenetic changes in cancer occur before genetic mutations. "If you can prevent methylation of those tumour suppressor genes, you might have a valuable prevention strategy," says Baylin.

Thus, if we see cancers when they are driven by methylation, then can we actually anticipate reversing the process by reversing the methylation changes. Thus, with prostate cancer can we anticipate a preventative measure as one increasing certain methylation preventative therapeutics, can we do the same with say MDS, and can we attempt to do the same with say a melanoma. This is what we examine herein.

What is methylation? Simply, the attachment of a methyl group to the cytosine molecule creates a methylated C. This is not a complicated process but one which happens frequently and may have significant effects. Cytosine gets methylated and is converted to 5-methyl cytosine. This is accomplished by means of two enzymes as depicted below. This occurs when we have a C and G adjacent. It occurs to the C in that pair. We depict that transition below. Note also that by using 5-Azacytadine we can block that transition.



From: Herman and Baylin, NEJM,

5-Methyl Cytosine

Now there are the CpG islands. These are C, cytosine, and G, guanine, adjacent nucleotides which are connected via a phosphodiester bone between the two, and multiple collections of these paired nucleotides. The CpG island is then an area dense in these CG pairs connected by the phosphodiester bond, but the "island" may contain nucleotides other than the CG pairs, but generally are high in CG pair concentration, usually more than 50%.

One should note that the statistical probability of such large CG pairings would normally be quite low. One would anticipate equal probability for any nucleotide and any nucleotide pairing. Furthermore, such a high concentration is statistically extremely rare but if often existentially quite common.

The CpG islands may be from 300 to over 3,000 base pairs in total length, and are frequently found in gene promoter regions. Thus, when the CpG islands are methylated, namely the C is methylated, then the island gets silenced as does the corresponding gene. Namely methylation of CpG islands can result in gene silencing. This then becomes a critical issue if the gene is a control gene such as PTEN, p53, or many of the critical pathway control genes. The CpG islands are also propagated to cell progeny during mitosis, Thus, a methylated island remains so in the cells progeny.

However, understanding methylation of islands, and having a means to demethylate the islands may present a reasonable way to develop therapeutics for cancers resulting from methylated regions. We shall examine that shortly.

As Laird and Jaenisch state:

The normal pattern of 5-methylcytosine distribution DNA methylation in mammals is found as a covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides. Most of the CpG dinucleotides in the human genome are methylated.

However, 5-methylcytosine makes up less than 1% of all nucleotides, since CpG dinucleotides are under-represented about five-fold in the mammalian genome. The paucity of CpG dinucleotides in the mammalian genome is attributed to a higher mutation rate of methylated versus unmethylated cytosine residues.

CpG dinucleotides and 5-methylcytosine are unevenly distributed in the genome. Most of the genome is heavily methylated with a corresponding deficit in CpG dinucleotides. About 1 to 2% of the genome consists of islands of non-methylated DNA and these sequences show the expected frequency of CpG dinucleotides.

CpG islands are about 1 kb long and are not only CpG-rich, but generally G/C-rich as well and are found at the 5' end of genes. All known housekeeping genes and some tissue-specific genes have associated CpG islands.

11.3.1 Methylation and Gene Expression

We now want to discuss methylation and gene expression. Reference will be made to the work of Herman and Baylin, Jones and Takai, McCabe et al, Allis et al, and Issa and Kantarjian.

We begin with Herman and Baylin and their description of the diagram below:

In most of the mammalian genome, which is depicted here as exons 1, 2, and 3 of a sample gene (boxes 1, 2, and 3), introns of the gene (line between the exons), and regions outside the gene, the CpG dinucleotide has been depleted during evolution, as shown by the small number of such sites (circles).

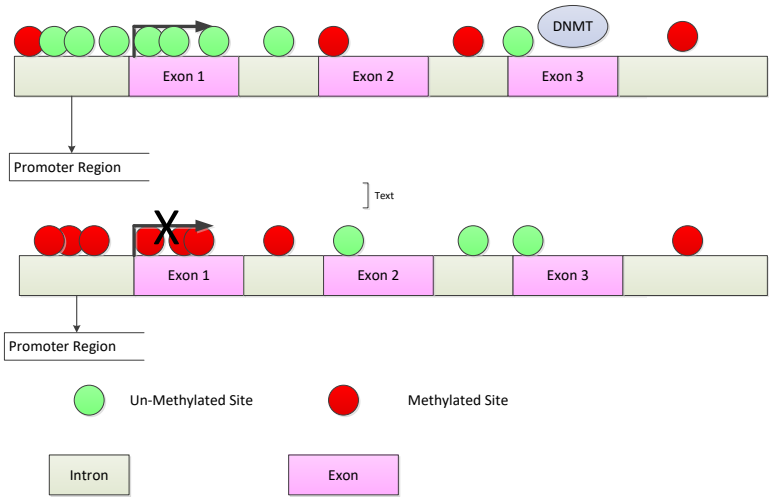
Small regions of DNA, approximately 0.5 to 4.0 kb in size, harbor the expected number of CpG sites and are termed CpG islands. Most of these are associated with promoter regions of approximately half the genes in the genome (numerous circles surrounding and within exon 1 of the sample gene). In normal cells, most CpG sites outside of CpG islands are methylated (black circles), whereas most CpG-island sites in gene promoters are unmethylated (white circles).

This methylated state in the bulk of the genome may help suppress unwanted transcription, whereas the unmethylated state of the CpG islands in gene promoters permits active gene transcription (arrow in upper panel). In cancer cells, the DNA-methylation and chromatin patterns are shifted.

Many CpG sites in the bulk of the genome and in coding regions of genes, which should be methylated, become unmethylated, and a growing list of genes have been identified as having abnormal methylation of promoters containing CpG islands, with associated transcriptional silencing (red X at the transcription start site).

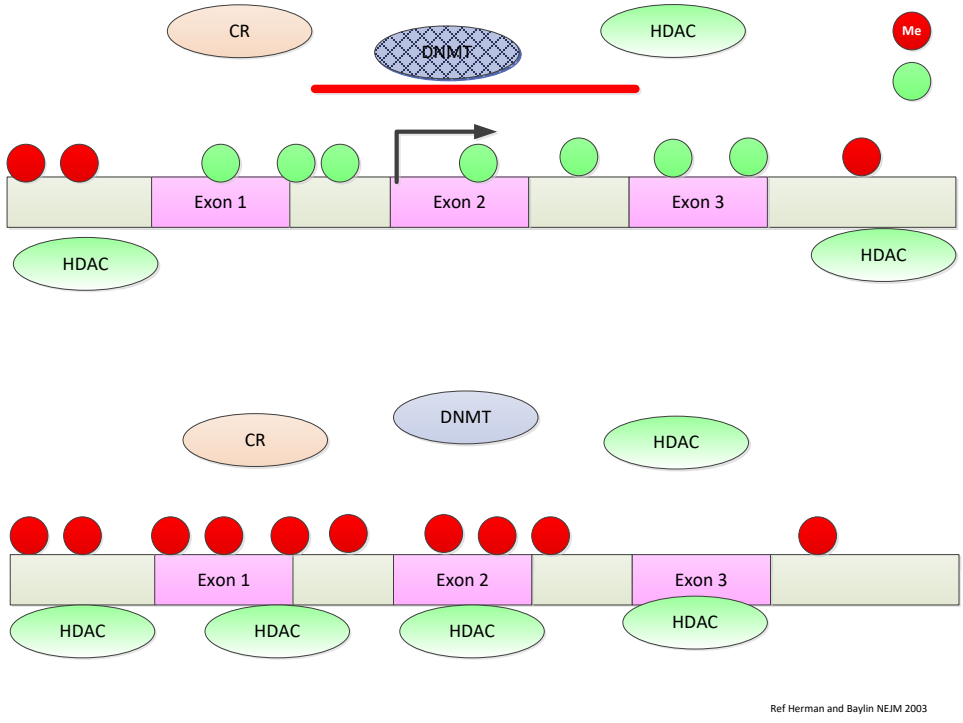
Although there are possible explanations and findings from ongoing investigations, it is not known why the DNA-methylating enzymes fail to methylate where they normally would and which of these enzymes are mediating the abnormal methylation of CpG islands in promoters.

We depict a modified version of their Figure below:



Thus, methylation in this case blocks the expression of the targeted gene.

Herman and Baylin also use the following Figure to describe more regarding methylation:



Ref Herman and Baylin NEJM 2003

As to the above they state:

The chromatin around the transcriptionally active (green arrow), unmethylated promoter is occupied by widely spaced nucleosomes composed of histone complexes in which key residues in the tails of histone H3 are in the acetylated state (green ovals), and those in the tails of histone H3 are methylated at lysine 4 (yellow asterisks).

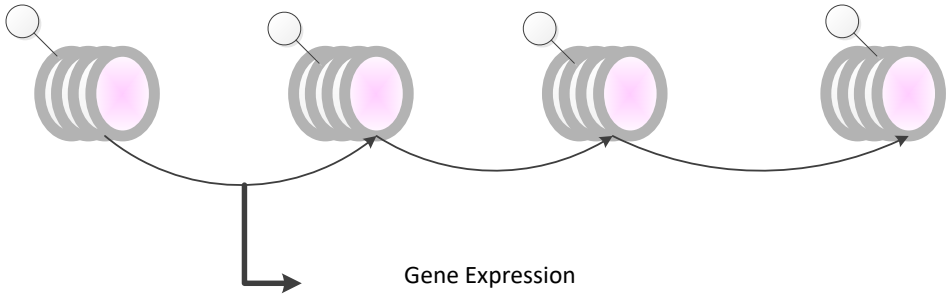
The region is accessible to key components of the gene-transcription apparatus, including primary transcription factors (TF); proteins with histone acetyltransferase activity (HAT), which maintain the histones in an acetylated state; and transcriptional coactivators (CA), which may also have histone acetyltransferase activities.

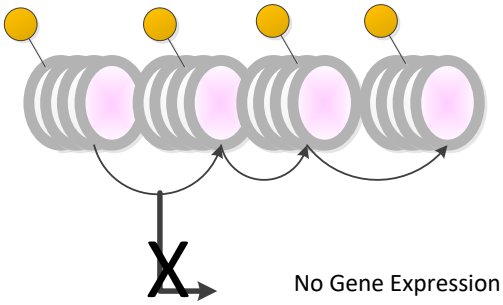
The flanking regions to either side of the unmethylated CpG island contain methylated cytosines. These regions are embedded in chromatin characteristic of transcriptionally silenced regions that is characterized by the binding of methylcytosine–binding proteins (MBPs) to the DNA methylated sites, and by nucleosomes that are more tightly compacted, with deacetylated histones (purple ovals) and methylated lysine 9 residues on the tails of histone H3 (black asterisks). The MBPs are part of complexes containing histone deacetylases (HDAC) that facilitate the deactivated state of the histones.

The blue vertical bars on either side of the unmethylated CpG island depict the molecular events, still to be determined, that prevent the spread of DNA methylation and of transcriptionally repressive chromatin across the CpG island in the promoter region of normal cells. The apparatus for DNA methylation, consisting of the DNA methyltransferases (DNMTs) and their complexes with transcriptional corepressors (CR) and histone deacetylases (HDAC), have access to the flanking areas but not to the CpG island in the promoter region within the barriers.

The lower panel depicts the breakdown of the barriers in a cancer cell, in which the transcriptionally repressive chromatin and DNA methylation have spread into the CpG island in the promoter region and correlate with transcriptional repression (red arrow with X) of the gene. The DNA-methylating complex now has access to the region, and the transcriptional machinery (transcriptional coactivators, histone acetyltransferase, and transcription factors) is excluded.

Now the histones may also be acetylated and drawn together. When histones are drawn closer the genes in between cannot be read and Thus, they are not expressed. We show that below:





Now we can summarize this as follows:

	Hypermethylated	Hypomethylated
Benign	Suppresses Proliferation Gene	Activates Suppressor Gene
Cancer	Suppresses Control Gene	Activates Proliferation Gene

What this shows is that methylation is good and bad. It is good if it suppresses the bad gene and bad if it suppresses a good gene, and vice versa.

11.3.2 Methylation and Deamination (C to T)

Methylation may also progress to more dramatic changes. We discuss here the change of C to T, a serious change in a DNA base pair which can result in dramatic changes in gene expression.

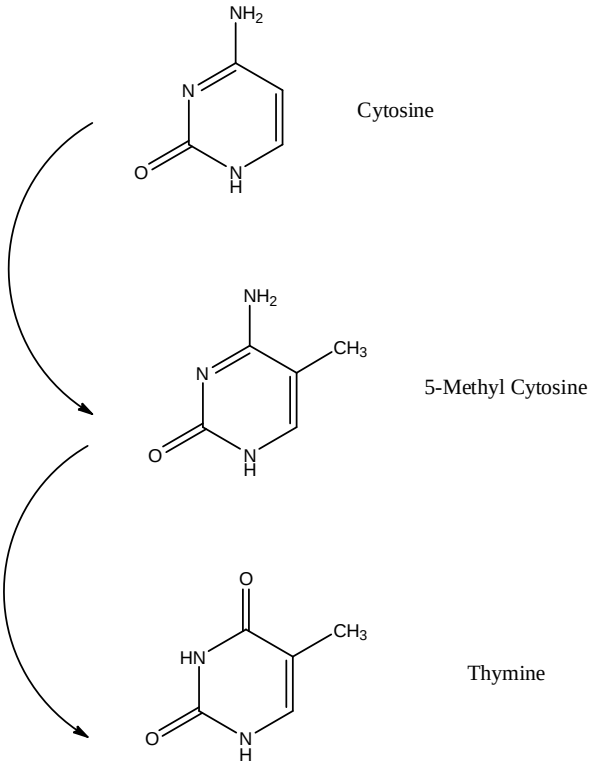
As Herman and Baylin state:

Although only four bases — adenine, guanine, cytosine, and thymine — spell out the primary sequence of DNA, there is a covalent modification of postreplicative DNA (i.e., DNA that has replicated itself in a dividing cell) that produces a "fifth base." Reactions using S -adenosyl-methionine as a methyl donor and catalyzed by enzymes called DNA methyltransferases (DNMTs) add a methyl group to the cytosine ring to form methyl cytosine.

In humans and other mammals, this modification is imposed only on cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide). The overall frequency of CpGs in the genome is substantially less than what would be mathematically predicted , probably because DNA methylation has progressively depleted the genome of CpG dinucleotides over the course of time.

The mechanism of the depletion is related to the propensity of methylated cytosine to deaminate, thereby forming thymidine. If this mutation is not repaired, a cytosine-to-thymidine change remains.

The depletion of CpG dinucleotides in the genome corresponds directly to sites of such nucleotide transitions, and this change is the most common type of genetic polymorphism (variation) in human populations.



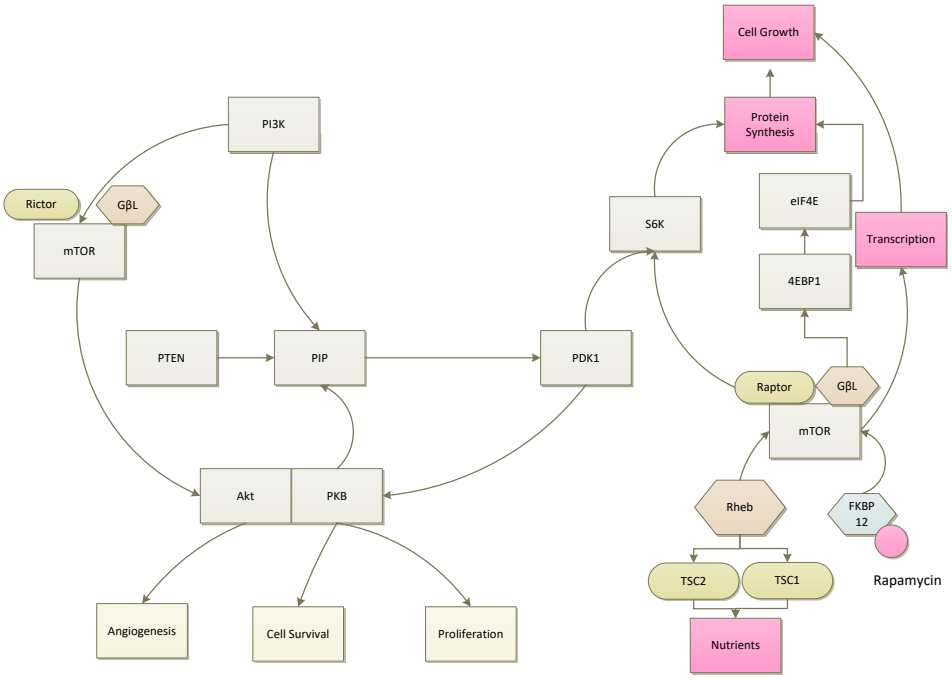
From Robertson (2001) we have some of the genes influenced by methylation or as he states:

CpG-island-associated genes involved in cell growth control or metastasis that can become hypermethylated and silenced in tumors.

We depict the Table below from Robertson on some of the genes impacted by this type of methylation. Most of these are significant regulatory genes.

Gene	*Function*
pRb	Regulator of G1/S phase transition
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds β-catenin, Regulation of actin cytoskeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell-cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTPI	Protects DNA from oxygen radical damage
0^6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metalloproteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by y interferon
p73	Apoptosis structurally similar to p53

For example, we show below some typical pathways and the above genes are seen targeted by methylation.



Methylation may then interfere with many of the genes in the above pathways.

11.3.3 Causes of Methylation

The major question which is often asked is what causes methylation. In Allis et al on p 460 the authors discuss some of the putative cause of methylation and methylation related cancers. Although not confirmative it is consistent with clinical correlations as well.

As Issa and Kartarjian state:

Much remains to be learned about the causes of DNA methylation abnormalities in cancer; for the most part, methylation seems to be gene specific. In some cases, a rare methylation event appears in cancer because of selection, while in others methylation anomalies are downstream of an oncogenic event …

As McCabe et al state:

DNA methylation patterns in human cancer cells are considerably distorted. Typically, cancer cells exhibit hypomethylation of intergenic regions that normally comprise the majority of a cell's methyl-cytosine content. Consequently, transposable elements may become active and contribute to the genomic instability observed in cancer cells.

Simultaneously, cancer cells exhibit hypermethylation within the promoter regions of many CpG island-associated tumor suppressor genes, such as the retinoblastoma gene (RB1), glutatione S-transferase pi (GSTP1), and E-cadherin (CDH1). As a result, these regulatory genes are transcriptionally silenced resulting in a loss-of-function. Thus, through the effects of both hypo- and hyper-methylation, DNA methylation significantly affects the genomic landscape of cancer cells, potentially to an even greater extent than coding region mutations, which are relatively rare

McCabe et al continue:

Although the precise molecular mechanisms underlying the establishment of aberrant DNA hypermethylation remain elusive, recent studies have identified some contributing etiologic factors.

For example, chronic exposure of human bronchial epithelial cells to ***tobacco-derived carcinogens drives hypermethylation*** *of several tumor suppressor genes including CDH1 and RASSF2A.*

Stable knockdown of DNMT1 prior to carcinogen exposure prevented methylation of several of these genes indicating a necessary role for this enzyme in the molecular mechanism underlying hypermethylation.

The reactive oxygen species (ROS) associated with chronic inflammation is another source of DNA damage with the potential to affect DNA methylation as halogenated pyrimidines, one form

of ROS-induced damage, mimic 5-methylcytosine and stimulate DNMT1-mediated CpG methylation in vitro and in vivo.

Indeed, study of the glutatione peroxidase 1 and 2 double knockout model of inflammatory bowel disease found that 60% of genes that are hypermethylated in colon cancers also exhibit aberrant methylation in the inflamed noncancerous precursor tissues. Although the mechanisms by which DNA damage mediates DNA methylation are not fully understood, O'Hagan and colleagues have examined the process with an engineered cell culture model in which a unique restriction site was incorporated into the CpG island of the E-cadherin promoter.

Thus, the actual molecular mechanics leading to methylation are not fully understood but like most cancers inflammation appears to be a driving factor. What the cause of that inflammation may be is not yet clear.

11.4 Methylation Effects on DNA

As is stated in the paper by Miranda and Jones:

DNA methylation is a covalent modification in which the 5₀position of cytosine is methylated in a reaction catalyzed by DNA methyltransferases (DNMTs) with S-adenosyl-methionine as the methyl donor.

In mammals, this modification occurs at CpG dinucleotides and can be catalyzed by three different enzymes, DNMT1, DMNT3a, and DNMT3b.DNAmethylation plays a role in the long-term silencing of transcription and in heterochromatin formation.

As an epigenetic modification, DNA methylation permits these silenced states to be inherited throughout cellular divisions.

We continue with the discussion in Mirand and Jones as follows:

Silencing of genetic elements can be successfully initiated and retained by histone modifications and chromatin structure. However, these modifications are easily reversible making them make poor gatekeepers for long-term silencing. Therefore, mammalian cells must possess an additional mechanism for prolong silencing of these sequences. An important component of this process is DNA methylation. DNA methylation is a stable modification that is inherited throughout cellular divisions.

When found within promoters, DNA methylation prevents the reactivation of silent genes, even when the repressive histone marks are reversed. This allows the daughter cells to retain the same expression pattern as the precursor cells and is important for many cellular processes including the silencing of repetitive elements, X-inactivation, imprinting, and development.

We now present a key Figure from Miranda and Joner regarding the methylated reading of DNA. They state regarding the Figure below:

Chromatin structure of CpG islands and CpG poor regions in healthy cells and during cancer. In healthy cells, CpG islands are generally hypomethylated. This allows for an open chromatin structure. However, the CpG poor regions found in repetitive elements within the intergenic and intronic regions of the genome are methylated and thereby maintain a closed chromatin structure. In cancer and on the inactive X chromosome many CpG islands become methylated, forcing these regions into a closed chromatin structure.

When CpG islands located within promoters are methylated, the corresponding genes are persistently silenced. In contrast, the CpG poor regions become hypomethylated allowing for an open chromatin structure.

As Robertson states:

It is now clear that the genome contains information in two forms, genetic and epigenetic. The genetic information provides the blueprint for the manufacture of all the proteins necessary to create a living thing while the epigenetic information provides instructions on how, where, and when the genetic information should be used.

Ensuring that genes are turned on at the proper time is as important as ensuring that they are turned off when not needed.

The major form of epigenetic information in mammalian cells is DNA methylation, or the covalent addition of a methyl group to the 5-position of cytosine predominantly within the CpG dinucleotide. DNA methylation has profound effects on the mammalian genome.

Some of these effects include transcriptional repression, chromatin structure modulation, X chromosome inactivation, genomic imprinting, and the suppression of the detrimental effects of repetitive and parasitic DNA sequences on genome integrity.

Robertson then proceeds to detail the genes impacted by hypermethylation. We summarize them below:

Gene	*Function*
pRb	Regulator of G1/S phase transition
p16 INK4a	Cyclin-dependent kinase inhibitor
p15 INK4b	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds b-catenin, Regulation of actin cyto-skeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell ± cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTP1	Protects DNA from oxygen radical damage
O6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metallo proteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by g interferon
p73	Apoptosis?, structurally similar to p53

Regarding PIN, the one which is most concern is the GSTP1 gene and its suppression allowing for DNA damage from inflammation and oxygenation damage.

In the context of cancer generation and progression, the epigenetic effect of hyper and hypo methylation is best described by Esteller:

The low level of DNA methylation in tumors as compared with the level of DNA methylation in their normal-tissue counterparts was one of the first epigenetic alterations to be found in human cancer.

The loss of methylation is mainly due to hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns −regions of DNA that allow alternative versions of the messenger RNA (mRNA) that are transcribed from a gene. A recent large-scale study of DNA methylation with the use of genomic microarrays has detected extensive hypo-methylated genomic regions in gene-poor areas.

During the development of a neoplasm, the degree of hypomethylation of genomic DNA increases as the lesion progresses from a benign proliferation of cells to an invasive cancer.

Three mechanisms have been proposed to ex-plain the contribution of DNA hypomethylation to the development of a cancer cell:

(i) generation of chromosomal instability,

(ii) reactivation of transposable elements, and

(iii) loss of imprinting.

Under methylation of DNA can favor mitotic recombination, leading to deletions and translocations, and it can also promote chromosomal rearrangements. This mechanism was seen in experiments in which the depletion of DNA methylation by the disruption of DNMTs caused

aneuploidy. Hypomethylation of DNA in malignant cells can reactivate intra-genomic endo-parasitic DNA.

1.1.1 Hypomethylation

We now consider the other extreme, hypomethylation. As Laird and Jaenisch state:

Hypomethylation: Reduced levels of global DNA methylation have been reported for a variety of malignancies in the past decade. Gama Sosa and coworkers found that in a wide variety of tumors, hypomethylation not only correlated with transformation, but also with tumor progression. In their analysis, only 7% of 43 normal tissues had 5-methylcytosine content below 0.8 mol%, whereas 10% of 21 benign tumors, 27% of 62 primary malignancies and 60% of 20 secondary malignancies had 5-methylcytosine content below 0.8 mol%. On the other hand, Feinberg and coworkers did not find a further reduction in DNA methylation levels in the progression from benign to malignant colonic neoplasia, suggesting an early role for DNA hypomethylation in colorectal cancer

1.1.2 Hypermethylation

As again with Laird and Jaenisch we have:

Hypermethylation: There have also been many reports of regional increases in DNA methylation levels. Baylin and coworkers have found regional hotspots for hypermethylation on chromosomes 3p, 11p and 17p in a variety of human tumors. These include CpG island areas that are normally never methylated in vivo, but are found to be methylated in tumor tissues. This is reminiscent of the changes that occur at CpG islands at non-essential genes in tissue culture.

Baylin's group has dissected the sequential order of hypermethylation events in an in vitro model for lung tumor progression. There is evidence for inactivation of tumor-suppressor gene function through hypermethylation of the Rb gene in sporadic retinoblastoma. Transient transfection experiments showed that specific hypermethylation in the promoter region of Rb could reduce expression to 8% of an unmethylated control. It is possible, therefore, that hypermethylation of tumor-suppressor genes leading to gene inactivation results in a selective growth advantage of the transformed cells.

From Issa, we have:

The mechanism whereby CpG island methylation suppresses gene transcription has been partially elucidated recently (Fig. 1), at least in vitro . Methylated CpG islands form excellent binding sites for methylated-DNA binding proteins (often with transcriptional repression properties), such as MeCp2. MeCp2 binding is followed by the recruitment of a protein complex that includes histone deacetylases (HDAC), and eventually leads to a closed chromatin configuration.

This closed chromatin configuration results in exclusion of transcription factors, Thus, insuring allele-specific inactivation. Methylation-related epigenetic silencing has also been found to be

associated with histone H3 lysine 9 (H3K9) methylation . Evidence suggests that H3K9 methylation is a critical modification that is associated with closed chromatin at DNA methylation sites, and it was proposed that a cascade of events follows DNA methylation (MeCP2 binding, H3K9 deacetylation, H3K9 methylation) and ensures transcriptional suppression (Fig. 1). Separately, DNMT1 can directly suppress transcription (without DNA methylation) through interactions with histone deacetylases . H3K9 methylation itself appears to set-up a silencing loop by attracting more DNA methylation , and may sometimes precede hypermethylation

11.4.1 Hypermethylation Induction

What starts the process of hypermethylation? What are its dynamics? Issa states:

There are complex changes in DNA methylation in cancer. For the most part, these changes involve simultaneous global demethylation, increased expression of DNMTs and de-novo methylation at previously unmethylated CpG islands. Demethylation was first discovered by studying overall 5-methy-cytosine (5mC) content in tumors, and appears to involve primarily satellite DNA, repetitive sequences, and CpG sites located in introns . The cause of this demethylation remains unclear, although it could be related to alterations in proliferation or cell-cycle control .

The functional consequences of hypomethylation are not entirely clear, but there is mounting evidence that gene-specific hypomethylation can cause increased expression of various genes that could contribute to the neoplastic phenotype . An increased mutation rate was demonstrated in cells in which severe hypomethylation (>75%) was achieved by homozygous deletion of DNMT1 , but it is not clear whether this degree of hypomethylation is ever achieved in neoplasms

Thus, we still have a great deal of work to fully understand these effects.

11.4.2 Hypermethylation and MDS

We now combine our understanding of methylation and that of MDS to provide insight on the relationship. There has been a great deal of recent literature on the impact of hypermethylation in MDS and we review some of the key contribution here. Issa presents a collection of aberrant CpG islands of hypermethylation found in MDS and we present his Table below:

Gene	*Methylation frequency (%)*	*Function*	*Note*
Calcitonin	50	Differentiation	
CDKN2B	23-80	Cyclin dependent kinase inhibitor; cell cycle/proliferation	Tumor-suppressor; methylation correlates with poor prognosis and progression to AML81
DAPK	50	Proapoptotic serine/threonine kinase	
RASS F1	9	Negative regulator of RAS signaling	Tumor-suppressor
FHIT	50	Purine metabolism	Putative tumor- suppressor; methylation correlates with poor prognosis and progression to AML68
HIC	32	Transcriptional repressor	Tumor-suppressor
CDH	15-27	Adhesion and motility	Methylation correlates with poor prognosis and progression to AML31
CTNNA	10	Alpha catenin	
ERα	7-19	Estrogen receptor	Methylation as part of a panel of genes (also including CDH1 and CDKN2A) correlates with poor prognosis and progression to AML
RIL	36-70	Proapoptotic, tumor- suppressor	
CDH13	21	Adhesion and motility	
NOR1	15	Oxidored-nitro domain- containing protein	
NPM2	20	Nucleophosmin/nucleoplasmin 2, involved in development	
OLIG2	41	Basic helix-loop-helix transcription factor	
PGRA	45	Progesterone receptor	
PGRB	45	Progesterone receptor	

With regards to the Table above Issa comments as follows:

Most studies of epigenetics in MDS have focused on DNA methylation so far. Several genes have been shown to be transcriptionally silenced in association with promoter DNA methylation in this disease. These include genes involved in cell-cycle regulation (CDKN2A), apoptosis (DAPK1, RIL), adhesion and motility (CDH1, CDH13) and others.

Separately, some of these genes clearly have minimal functional impact on the disease, not being expressed in normal hematopoietic cells. MDS cases often show hypermethylation of several genes simultaneously. Thus, hypermethylation can be viewed in a similar way as mismatch repair deficiency and microsatellite instability in cancer: many loci are affected simultaneously, a few of which likely have functional consequences.

In MDS, CDKN2B (P15) has been the most extensively studied gene.

CDKN2B was reported to be methylated in 30-80% of the cases, with the variability being likely due to different methods of measurement, as well as inclusion of different types of MDS. Thus, CDKN2B methylation has been reported to be very frequent in therapy related MDS, as well as in CMML, in RAEB-T or AML arising from MDS . CDKN2B methylation in MDS has also been associated with older age, deletions of 5q and 7q, and a poor prognosis . Interestingly, when

present, CDKN2B methylation in MDS has been shown to affect multiple lineages from clonogenic cells to circulating mononuclear cells .

In a mouse model, loss of CDKN2B was associated with enhanced myeloid progenitor and reduced erythroid progenitor formation , suggesting that its inactivation plays a functional role in MDS.

The interplay between hypermethylation and gene suppression is complex but as we have shown above from Issa it is quite prevalent. As Jiang et al state:

Myelodysplastic syndromes (MDSs) are clonal hematologic disorders that frequently represent an intermediate disease stage before progression to acute myeloid leukemia (AML). As such, study of MDS/AML can provide insight into the mechanisms of neoplastic evolution. In 184 patients with MDS and AML, DNA methylation microarray and high-density single nucleotide polymorphism array (SNP-A) karyotyping were used to assess the relative contributions of aberrant DNA methylation and chromosomal deletions to tumor-suppressor gene (TSG) silencing during disease progression.

Aberrant methylation was seen in every sample, on average affecting 91 of 1505 CpG loci in early MDS and 179 of 1505 loci after blast transformation (refractory anemia with excess blasts [RAEB]/AML). In contrast, chromosome aberrations were seen in 79% of early MDS samples and 90% of RAEB/AML samples, and were not as widely distributed over the genome. Analysis of the most frequently aberrantly methylated genes identified FZD9 as a candidate TSG on chromosome 7. In patients with chromosome deletion at the FZD9 locus, aberrant methylation of the remaining allele was associated with the poorest clinical outcome.

These results indicate that aberrant methylation can cooperate with chromosome deletions to silence TSG. However, the ubiquity, extent, and correlation with disease progression suggest that aberrant DNA methylation is the dominant mechanism for TSG silencing and clonal variation in MDS evolution to AML.

Tumor Suppressor Gene silencing is a significant if not the dominant factor. As has been discussed elsewhere, the cell has a complex control mechanism to ensure that uncontrolled proliferation.

11.5 Demethylating Therapeutics

Myelodysplastic Syndrome is an uncommon hematological cancer mostly caused by excess exposure to radiation, chemicals such as benzene, and insecticides. The specific genetic causes are still a work in progress. However, there is certain therapeutics which addresses some of the pathway aberrancies which characterize the disease, specifically hypermethylation.

11.5.1 Epigenetic Control Paradigms

As Taferri and Vardiman state:

According to the 2008 World Health Organization (WHO) classification system for hematologic cancers, the primary myelodysplastic syndromes are one of five major categories of myeloid neoplasms. The main feature of myeloid neoplasms is stem-cell–derived clonal myelopoiesis with altered proliferation and differentiation. The phenotypic diversity of these neoplasms has been ascribed to different patterns of dysregulated signal transduction caused by transforming mutations that affect the hematopoietic stem cell.

*There is increasing evidence that haploinsufficiency, **epigenetic changes**, and abnormalities in cytokines, the immune system, and bone marrow stroma all contribute to the development of the myelodysplastic syndromes.*

Thus, MDS is both complex in presentation and complex in development. Melanoma and prostate cancer are more clearly characterized morphologically and generally in genetic development. The presentation may involve the white cells, red cells or platelets, or any combination thereof. It is often discovered as an incidental finding on a blood test with lowered amounts of one or several of the constituents. If it has progressed more it may also present in the bone biopsy with more than normal blasts, immature cells.

As DeVita et al state:

Myelodysplastic syndromes (MDSs) are a group of complex and heterogeneous clonal hematopoietic stem cell disorders whose defining characteristics are dysplasia of one or several hematopoietic cell lineages, hypercellular marrows, and blood cytopenias.

1 Although historically considered as a preleukemic state, most patients with MDS do not transform into an acute myeloid leukemia (AML), but will instead succumb to complications of persistent cytopenias. Indeed, the pathophysiology of MDS extends from immune-mediated mechanisms and excessive apoptosis resulting in marrow failure to arrest of maturation and proliferation resembling the mechanisms at play in AML.

2 The diverse pathophysiology of factors that contribute to the development of MDS is reflected in vast differences of patients' prognosis, which is increasingly recognized and reflected in the design of more elaborate systems of diagnosis, classification, and prognostication.

Let us begin with a simple set of statements regarding the micro RNA elements which are often seen at the heart of the disease. As Croce states:

Several of the miRNAs that have been described as suppressors have been found to be deleted or mutated in various human malignancies. For example, loss of miR-15a and miR-16-1 has also been observed in prostate cancer and multiple myeloma (TABLE 1). Members of the miR-29 family have been found to be deleted in a fraction of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients.

As Croce further states:

MicroRNAs as targets of epigenetic changes. The most studied epigenetic changes in cancer cells are the methylation of cytosines in the dinucleotide CpG in DNA62. Such 'methylable' sites, known as CpG islands, are preferentially located in the 5' region (which consists of the promoter, 5' uTR and exon 1) of many genes, are non-methylated in normal cells and are transcribed in the presence of the appropriate transcription factors. Methylation of the CpG islands of tumour suppressors results in their silencing and contributes to malignant transformation.

As mentioned above, the expression of miRNAs can be affected by genetic changes, such as deletion, gene amplification and mutation, and by transcription factors. In addition, the expression of miRNAs can be affected by epigenetic changes, such as methylation of the CpG islands of their promoters. Saito et al. reported that miR-127 is silenced by promoter methylation in bladder tumours and that its expression could be restored by using hypomethylating agents such as azacitidine.

This miRNA targets BCL6, an oncogene that is involved in the development of diffuse large b cell lymphoma. Therefore, the silencing of miR-127 may lead to the overexpression of bCL6. Other investigators have described additional miRNAs that are silenced by methylation in various cancers and that can be reactivated by hypomethylating agents.

As Das and Singal state:

Hypermethylation is associated with many leukemias and other hematologic diseases. *Many genes, such as the calcitonin gene, p15INK4B, p21Cip1/Waf1, the ER gene, SDC4, MDR, and so on, were seen to be hypermethylated in a variety of hematologic cancers.*

The calcitonin gene and p15 were hypermethylated in 65% of myelodysplastic syndromes*, and it was found that p15 methylation at diagnosis was associated with lower survival and transformation to acute myeloid leukemia.*

Also, acquisition of p15 methylation at a later date signaled disease progression. These may suggest the role of p15 as a marker of leukemic transformation. Acute myeloid leukemia demonstrated frequent hypermethylation of ER, MYOD1, PITX2, GPR37, and SDC4

Thus, MDS is closely related to methylation, and in effect is caused by methylation. In addition, as we show below its management is also performed through an understanding of methylation and managing that process.

From DeVita et al (pp 479-480) we have:

Originally synthesized as cytotoxic antimetabolite drugs in the1960s, 21azacytosine nucleosides were recognized as inhibitors of DNA methylation in the early 1980s. 5-Azacitidine (5AC) and 2-deoxy-5-azacitidine induced muscle, fat, and chondrocyte differentiation in mouse embryo cells, in association with reversal of DNA methylation. Incorporation of azacytosine nucleosides into DNA in lieu of cytosine residues was shown to be associated with inhibition of DNMT activity. DNMT inhibition requires incorporation of DAC triphosphate into DNA in lieu of cytosine

residues. The incorporated azacytosine nucleoside forms an irreversible inactive adduct with DNMT.

Sequential reversal of DNA methylation results when DNA replication proceeds in absence of active DNMT. 5AC must be dephosphorylated and converted to DAC diphosphate by ribo-nucleotide reductase before it can be activated through triphosphorylation; DAC does not require the ribo-nucleotidereductase. 5AC can also be incorporated into RNA; this inhibits tRNA cytosine methyltransferase.

This may contribute to an inhibition of protein synthesis. The azacytosine nucleosides exhibit complex dose-response characteristics. At low concentrations (0.2 to 1 mcM), the "epigenetic" activities of these drugs predominate, with reversal of DNA methylation and induction of terminal differentiation in some systems. As concentrations are increased, apoptosis be-comes more prominent.

Cell lines with 30-fold resistance to the cytotoxic effects of DAC continue to reverse methylation in response to this nucleoside, suggesting that the methylation re-versing and cytotoxic activities of this compound can be sepa-rated.27The ability of these drugs to inhibit cell cycle, at least in part through induction of p21WAF1/CIP1expression, complicates the goal of reversing DNA methylation because the latter re-quires DNA replication with the azacytosine nucleoside incorporated into the DNA.

The two azacytosine nucleosides in clinical use are highly unstable in aqueous solution. In aqueous solutions, the drugs readily hydrolyze and inactivate.28In clinical practice, the drugs must be administered shortly after reconstitution. The drugs are also metabolized by cytidine deaminase, leading to a short half-life in plasma.

Thus, there have been significant developments in methylation control. Recent papers by Blum and by Lubbert et al discuss some of the therapeutic issues as well.

11.5.2 Azacitidine and Decitabine and MDS

Understanding the impact of methylation in MDS recent efforts have led to certain therapeutics which have been of help.

As Issa and Kantarjian state:

Two nucleoside inhibitors of DNA methylation, azacitidine and decitabine, are now standard of care for the treatment of the myelodysplastic syndrome, a deadly form of leukemia. These old drugs, developed as cytotoxic agents and nearly abandoned decades ago were resurrected by the renewed interest in DNA methylation.

They have now provided proof of principle for epigenetic therapy, the final chapter in the long saga to provide legitimacy to the field of epigenetics in cancer. But challenges remain; we don't understand precisely how or why the drugs work or stop working after an initial response.

Extending these promising findings to solid tumors faces substantial hurdles from drug uptake to clinical trial design.

We do not know yet how to select patients for this therapy and how to move it from life extension to cure. The epigenetic potential of DNA methylation inhibitors may be limited by other epigenetic mechanisms that are also worth exploring as therapeutic targets. But the idea of stably changing gene expression in vivo has transformative potential in cancer therapy and beyond.

They continue:

Drugs that inhibit DNA methylation were discovered by pure serendipity . Cytosine analogs developed as cytotoxic anticancer agents in the 1960s and tested in the clinic in the 1970s were found to induce peculiar differentiation phenotypes in vitro (16). This DNA hypomethylating property is limited to cytosine analogs with modifications of the ring. Other cytosine or nucleoside analogs do not affect DNA methylation directly. Eventually, this property of the two main analogs, 5-azacytidine (AZA) and 5-aza-deoxycytidine (DAC), was traced to their ability to incorporate into DNA, trap DNA methyltransferases (DNMTs), and target these enzymes for degradation. DNA synthesis in the absence of these enzymes then results in hypomethylation in the daughter cells and eventually to reactivation of silenced gene expression. Several other modified nucleoside analogs have been described either in preclinical studies or in early stage clinical trials.

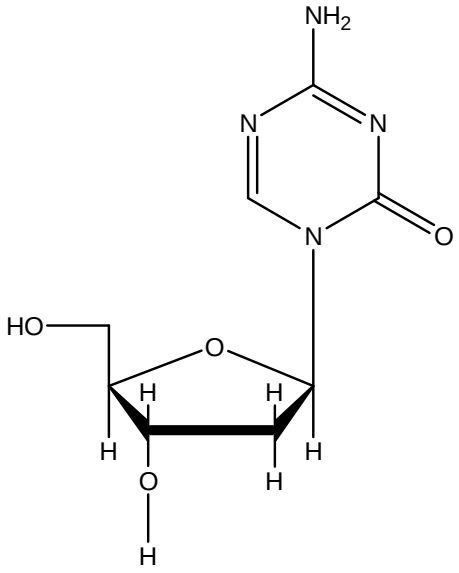
As Li has stated:

The strategies targeting DNA methylation. Epigenetic control of gene expression by DNA methylation has a great impact on cell proliferation and differentiation. Hypermethylation of promoter regions results in specific suppression of gene expression, including the expression of tumor suppressors, which could promote cancer development.

Conversely, demethylation of DNA may enhance cell apoptosis or reduce cell growth. This concept has been proven by a recently approved anticancer drug decitabine for the treatment of myelodysplastic syndrome. Decitabine (Dacogen; MGI Pharma) is a nucleoside analogue that inhibits DNA methylation.

It demethylates the p73 promoter and induces reexpression of p73, Thus, activating the caspase cascade and leading to leukemic myeloid cell death.26 DNA hypermethylation in tumor cells may be involved in resistance to interferon (INF)-induced apoptosis, and inhibition of DNA methylation may also enhance the therapeutic effect of INF. Treatment of cancer cells with specific DNA demethylating nucleoside analogue was shown to augment the effect of INF.

Now decitabine is shown below in detail. It is a cytosine derivative with several modifications. It functions in a manner similar to azacitidine. We have discussed that previously.



From Boumber et al we have the following regarding therapeutics for epigenetic drugs:

What Is Epigenetic Therapy? The understanding that epigenetic changes are prevalent in cancer and play a causative role in its biology has led to the development of new therapeutic approaches that target the epigenetic machinery. The first successful drugs developed as epigenetic agents were DNA methyltransferase inhibitors; these were followed by histone deacetylase inhibitors (HDIs).

Both classes of drugs aim at reversing gene silencing and demonstrate antitumor activity in vitro and in vivo. Several other classes of drugs have been developed that target various other components of the epigenetic machinery; one such class is the histone methyltransferases, with new drugs in this class currently in early preclinical development

The authors continue:

What Has Been Done? The inhibitors of DNA methylation used clinically are nucleoside analogues that get converted into deoxy-nucleotide-triphosphates (dNTPs) and become incorporated into DNA in place of cytosine during DNA replication. They trap all DNA methyltransferases and target them for degradation. At low doses these drugs do not inhibit proliferation; they reactivate gene expression and have shown clinical activity as anticancer agents.

Azacitidine was the first hypomethylating agent approved by the FDA; its approval, in 2004, for the treatment of myelodysplastic disorders and leukemia, was followed by the approval, in 2006, of decitabine. Both drugs produce remissions or clinical improvements in more than 30% of patients treated. Features of responses have included the requirement for multiple cycles of therapy, slow response, and relatively few side effects.

On the molecular level, demethylation, gene reactivation, and clonal elimination were observed in treated patients. The data in myelodysplastic syndrome (MDS) represent a proof-of-principle for epigenetic therapy for cancer, in particular in myeloid disorders.

From Boumber et al we have the following Table of many of the recent therapeutics:

Drug Class	Compound
DNMT Inhibitor	Azacitidine
	Decitabine
	S110
	CP-400
	Nanaomycin
HDAC Inhibitor	Vorinostat
	Romidepsin
	Panobinostat
	Valproic Acid
	Belinostat
HMT Inhibitor	Deazaneoplanocin
	Quinazoline
	Ellagic Acid
Histone demethylase inhibitor	Polyamine analogues
	Hydroxamate analogs
GAT inhibitor	Spermidinyl
	Hydrazinocurcumin
	Pyrazolone

As Stressman et al state:

Aberrant DNA methylation patterns play an important role in the pathogenesis of hematologic malignancies.

The DNA methyltransferase inhibitors azacytidine and decitabine have shown significant clinical benefits in the treatment of myelodysplastic syndrome (MDS), but their precise mode of action remains to be established. Both drugs have been shown the ability to deplete DNA methyltransferase enzymes and to induce DNA demethylation and epigenetic reprogramming in vitro. However, drug-induced methylation changes have remained poorly characterized in patients and therapy-related models.

We have now analyzed azacytidine-induced demethylation responses in myeloid leukemia cell lines. These cells showed remarkable differences in the drug-induced depletion of DNA methyltransferases that coincided with their demethylation responses. In agreement with these data, DNA methylation analysis of blood and bone marrow samples from MDS patients undergoing azacytidine therapy also revealed substantial differences in the epigenetic responses of individual patients.

Significant, transient demethylation could be observed in 3 of 6 patients and affected many hypermethylated loci in a complex pattern. Our results provide important proof-of-mechanism data for the demethylating activity of azacytidine in MDS patients and provide detailed insight into drug-induced demethylation responses.

11.5.3 Environmental and Genetic Causes and Factors

The main problem with MDS is that there is not clear genetic pathway and causal relationship. As DeVita et al state:

No etiologic factor is identified in most patients with MDS. MDS is more frequent in men than women by a factor of 1.8. It has been associated with smoking and hair dyes, exposure to agricultural and industrial toxins, drugs (e.g., chloramphenicol), and occupational exposures to stone and cereal dusts. MDS has been associated with exposure to ionizing radiation (atomic bomb survivors in Japan, decontamination workers following the Chernobyl nuclear plant accident) and chronic exposure to low-dose radiation (radiopharmaceuticals). Some inherited hematologic disorders (Fanconi anemia, dy-skeratosis congenita, Shwachman-Diamond syndrome, Diamond- Blackfan syndrome) are also associated with a higher risk of MDS.

Thus, there is no clear causal factor or factors recognized at this time.

In a recent paper by Suzuki et al the authors discuss some of the causes of methylation and in turn cancers. They state:

Evidence now suggests that epigenetic abnormalities, particularly altered DNA methylation, play a crucial role in the development and progression of human gastrointestinal malignancies. Two distinct DNA methylation abnormalities are observed together in cancer.

One is an overall genome-wide reduction in DNA methylation (global hypomethylation) and the other is regional hypermethylation within the CpG islands of specific gene promoters. Global hypomethylation is believed to induce proto-oncogene activation and chromosomal instability, whereas regional hypermethylation is strongly associated with transcriptional silencing of tumor suppressor genes.

To date, genes involved in regulation of the cell cycle, DNA repair, growth signaling, angiogenesis, and apoptosis, are all known to be inactivated by hypermethylation. Recently developed techniques for detecting changes in DNA methylation have dramatically enhanced our understanding of the patterns of methylation that occur as cancers progress. One of the key contributors to aberrant methylation is aging, but other patterns of methylation are cancer-specific and detected only in a subset of tumors exhibiting the CpG island methylator phenotype (CIMP).

Although the cause of altered patterns of DNA methylation in cancer remains unknown, it is believed that epidemiological factors, notably dietary folate intake, might strongly influence DNA methylation patterns.

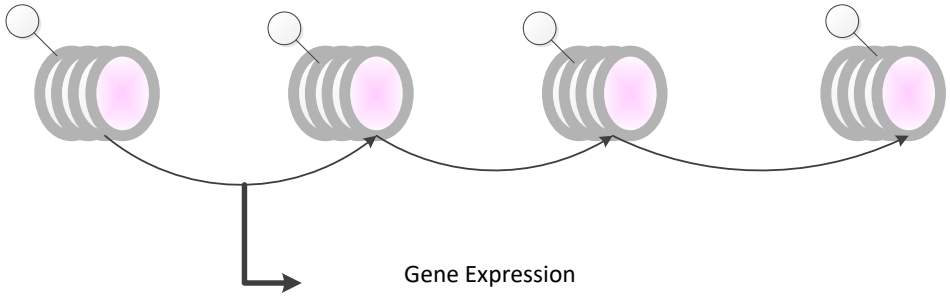
Recent studies further suggest that polymorphisms of genes involved in folate metabolism are causally related to the development of cancer.

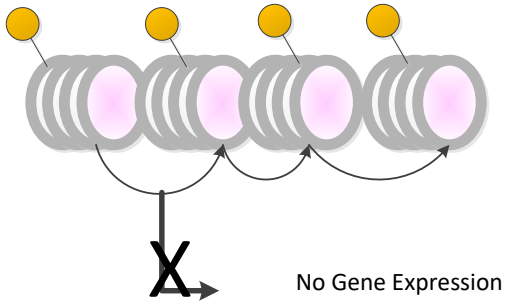
11.5.4 Acetylation and Histones

Histones also play a role in the control of genes and their expression. Whereas we can have methylated or non-methylated CpG regions we can have acetylated histones which can have another layer of control on gene expression. It is not us intend to discuss this in detail but merely to point out its significance.

11.5.5 DNA and Histones

Let us consider the DNA as it is wrapped around histones, and how it may wind out or become more closely packed. We demonstrate this below.





11.5.6 Deacetylation Therapeutics

From p 481 in DeVita et al:

The increasing recognition of the critical importance of his-tone modifications in regulating the transcriptional permissivity of chromatin has led to intense interest in compounds that can inhibit the activity of HDAC proteins, facilitating acetylation of lysines associated with

transcriptional activation of genes. The first generation of HDAC inhibitors were small chain fatty acids, including sodium butyrate, arginine butyrate, sodium phenylbutyrate, and valproic acid.

These agents require sub millimolar to millimolar concentrations to inhibit HDACs. Like the DNMT inhibitors, these compounds have complex pharmacodynamic properties. At the lowest concentrations associated with HDAC inhibitory activity, these com-pounds may increase cellular proliferation. At high concentrations, cell cycle arrest occurs, associated with induction of p21WAF1/CIP1and evidence of differentiation.

At concentrations exceeding 1 mM, apoptosis is induced. Second-generation HDAC inhibitors include hydroxamic acids, cyclic depsipeptides, and benzamides. The hydroxamic acid HDAC inhibitors include vorinostat (suberoylanilide hydroxamic acid, SAHA), which was synthesized as a derivative of the differentiation inducer hexamethylene bisacetamide.

Other hydroxamic acid HDAC inhibitors under clinical investigation include belinostat (PXD1010)48and LBH589.Hydroxamic HDAC inhibitors fit into and interact with the catalytic core of HDACs. Hydroxamic acids inhibit HDACs of class I and II.51Romidepsin (FK228) is a depsipeptide with potent HDAC inhibitory activity. Romidepsin requires reduction for optimal activity and appears to specifically inhibit class I HDACs.52The benzamide HDAC inhibitors include SNDX 275 (formerly known as MS275),CI994, and MGCD0103 are selective for class I HDACs. Many proteins in addition to histones serve as substrates for protein acetylases and can Thus, be impacted by HDAC inhibitors. These include transcription factors such as p53,E2F1, and GATA 1.DNA binding proteins such as HMG-158and tubulin can also be acetylated by acetyl transferases.

Protein acetylation can result in increased DNA binding, impact protein-protein interactions, and increase protein stability.

Given that a wide variety of proteins can undergo acetylation in the presence of HDAC inhibitors, it is not surprising that administration of HDAC inhibitors has been associated with a panoply of effects on cellular physiology. As predicted, administration of HDAC inhibitors induces alterations in gene expression. This includes both up and down regulation. Expression profiling has suggested that between 2% and 10%of genes studied may have their expression altered by exposure to HDAC inhibitors; however, the number of genes whose expression is reliably altered in a number of different cancer cell lines in response to a variety of HDAC inhibitors is few.

11.5.7 Bone Marrow Transplants

One of the most successful techniques used in many hematopoietic malignancies is bone marrow transplants (“BMT”). Allogeneic transplants, with HLA matches being high, are often the only techniques which and effectively restart the system and allow for regrowth of a stable hematopoietic system. We briefly discuss BMT as applied to MDS. The intent is not to provide any substantial detail on BMT but to allow for an understanding of its place in the treatment of MDS.

11.6 Basic Principles

BMT is the ultimate approach, despite the ability to reduce methylation with azacytidine, it is not curative. As Sekeres in a summary article states:

Those who treat patients with myelodysplastic syndromes (MDS) have been forced to become comfortable with a rather uncomfortable truth. MDS is a bone marrow failure syndrome that represents the most commonly diagnosed myeloid malignancy and predominantly affects older adults, with a median age at diagnosis of 71 years.

The only cure for MDS is hematopoietic stem-cell transplantation (HSCT). For a variety of reasons, including patient comorbidities, availability of related or matched donors, related donor comorbidities, physician and patient preference, and treatment-related adverse events, transplantation is only considered in approximately 5% of patients with MDS.

Thus, even when we offer disease-modifying therapies such as azacitidine, decitabine, and lenalidomide, we are ultimately palliating 95% of our patients. Despite this, patients often perceive these drugs to have curative potential in this setting, but cure is unfortunately not possible with these agents.

As DeVita et al state (note, they use SCT, stem cell transplants, for our use of BMT, and the difference for this purpose is minimal):

Allogeneic SCT (stem cell transplants) remains the only treatment modality that can lead to long-term disease-free survival. Given the demographics of MDS, only few patients will ultimately benefit from SCT. Treatment-related morbidity and mortality remain substantial impediments to SCT. SCT with reduced intensity conditioning can decrease the toxicity of the procedure, but at the cost of higher relapse likelihood. Matched unrelated donor transplants may overcome some of the shortage of suitable donors. Although effective, they carry a higher risk of toxicities. Judicious selection of patients for SCT is therefore crucial, particularly in the context of therapies such as lenalidomide or DNMT inhibitors.

Outcome is generally most favorable in patients who may need transplant the least, such as younger patients with low-risk MDS. In a study by the International Bone Marrow Transplant Registry, 452 recipients of HLA-identical sibling transplants with a median age of 34 years and high-risk MDS in two-thirds, overall survival at 3 years was 42%. Survival was more favorable with young age and platelet counts less than 100 × 109/L. Relapse was highest in patients with high percentages of marrow blasts at transplantation, with high IPSS scores, and with T-cell depleted transplants. Diseases-free survival was 60% in the low-risk, 36% in the intermediate-1, and 28% in intermediate-2 risk groups. This compared to 5-year survival rates of 55%, 35%, and 7%, respectively, for unselected patients not receiving SCT, suggesting a benefit of SCT mostly for high-risk MDS patients.

A key issue remains the optimal timing of SCT. Using a Markov decision model, three transplant strategies were compared: (1) SCT at diagnosis, (2) SCT at the time of progression to leukemia;

and (3) SCT sometime after diagnosis but prior to leukemic progression.63 Delaying transplant was most beneficial for patients in the low and intermediate-1 IPSS groups, an effect that was more noticeable in patients younger than 40 years. Earlier transplantation, on the other hand, improved survival in the intermediate-2 and high IPSS groups.

11.6.1 Efficacy

One of the recent putative studies was by Koreth et al which state:

Erythropoiesis-stimulating agents may offer a survival advantage for anemic patients or those with RBC transfusion-dependent low/ intermediate-1 IPSS MDS.22,23 Hypomethylating agent therapy can reduce rate of AML progression in patients with intermediate-2/high IPSS MDS, and azacytidine has been demonstrated to improve survival.24-26 Unfortunately these treatments seldom induce durable remissions, and none are curative. Allogeneic hematopoietic stem-cell transplantation is potentially curative.

In myeloablative conditioning (MAC) transplantation, IPSS risk is correlated with MDS relapse and disease-free survival.27 Treatment-related mortality (TRM) is 35% to 80%, varying with age and other factors. In a prior analysis, we documented that for patients 18 to 60 years of age with intermediate- 2/high IPSS MDS, early MAC transplantation provides maximal quality-adjusted survival.

However, 75% of patients with MDS are 60 years at diagnosis and are typically not considered MAC transplantation candidates.

In patients 60 years of age, reduced-intensity conditioning (RIC) transplantation is potentially curative but is also associated with mortality risk. Retrospectively, TRM was 26% to 41%, with long-term MDS/AML survival of 27% to 54%.

RIC transplantation in older patients remains uncertain because MDS prognosis differs from that of younger patients, and RIC and MAC transplantation risks and benefits may also differ. A retrospective report suggests that transplantation benefits patients with advanced MDS/AML who are 60 to 70 years old, but head-to-head comparisons of RIC transplantation versus nontransplantation approaches are lacking for MDS.

Koreth et al conclude:

For 223 patients with intermediate-2/high IPSS, early RIC transplantation had an LE of 36 months versus 28 months with nontransplantation therapy. … a Kaplan- Meier plot derived from the Monte Carlo simulation. QoL inclusion also indicated QALE benefit with early RIC transplantation, and sensitivity analyses supported RIC transplantation as a preferred option across the range of plausible state utilities for patients with intermediate-2/high MDS receiving hypomethylating agent therapy (0.33 to 0.73) versus the range of plausible state utilities after RIC transplantation (0.6 to 0.92). Explicitly modeling a plateau of long-term post-transplantation survival or discounting future survival also did not change the conclusion

….In conclusion, we undertook decision modeling to quantify benefit of RIC transplantation versus non-transplantation therapies in patients with de novo MDS aged 60 to 70 years. We conclude that early RIC transplantation offers survival benefit for intermediate-2/high IPSS MDS, but not for low/intermediate-1 IPSS MDS. These simple but robust findings may help clinical decision making for the older patient with MDS.

Sekeres comments on the above as follows:

In the article that accompanies this editorial, Koreth et al9 report on a Markov decision analysis exploring the role of reduced-intensity allogeneic HSCT in older patients with MDS. This statistical technique relies on assumptions, which themselves are based on best estimates of outcome given in previously published studies, to play out scenarios of what would happen in real life to a given patient if he or she decided to undergo HSCT early, at or near diagnosis, or instead to pursue supportive care, growth factor, or disease-modifying therapy.

Although this approach is not perfect, it does allow for sensitivity analyses in which assumptions can be changed to see if the same conclusion holds, and it is the best substitute available in the absence of prospective, randomized studies….

The analysis by Koreth et al9 addresses these shortcomings. Now, given the non myeloablative preparative regimen, the median age of the 132 patients undergoing transplantation gleaned from the Center for International Blood and Marrow Transplant Research, Dana- Farber Cancer Institute, and Fred Hutchinson Cancer Research Center data sets is 64 years—closer to what we see in clinic. Patients who did not undergo transplantation included 132 with lower-risk disease (IPSS low and intermediate-1) receiving best supportive care; 91 anemic or transfusion-dependent patients receiving erythropoiesis stimulating agents; and 164 higher-risk patients with MDS receiving azacitidine or decitabine.

Patients being treated with lenalidomide, immunosuppressive approaches, or drug combinations were not included. Primary end points of the model were life expectancy (LE) and quality-adjusted life expectancy, an end point adjusted for quality of life, the values of which were derived from studies in which patients may not reflect those included in the current analysis.

The authors tried to keep the assumptions used in an already complicated model to a minimum, and in so doing ignored some real-life scenarios, such as a patient initially in the non-transplantation arm deciding at a later time to undergo transplantation.

11.6.2 Immunotherapy

The third general step is the use of CIK, or cytokine induced killer cells. These are somewhat akin to NK cells and have been developed specifically for cancers of these type. We briefly discuss how they are prepared. The efficacy is yet to be fully determined but there is a large base of Phase I and II Trials demonstrating efficacy.

Lin and Hui provide a definition for CIK cells:

Cytokine-induced killer (CIK) cells are polyclonal T effector cells generated when cultured under cytokine stimulation. CIK cells exhibit potent, non-MHC-restricted cytolytic activities against susceptible tumor cells of both autologous and allogeneic origins. Over the past 20 years, CIK cells have evolved from experimental observations into early clinical studies with encouraging preliminary efficacy towards susceptible autologous and allogeneic tumor cells in both therapeutic and adjuvant settings. … we anticipate that the continuous therapeutic application of CIK cells will likely be developed along two major directions: overcoming the challenge to organize large prospective randomized clinical trials to define the roles of CIK cells in cancer immunotherapy and expanding its spectrum of cytotoxicity towards resistant tumor cells through experimental manipulations.

Jiang et al add to this description as follows:

The number of immune cells, especially dendritic cells and cytotoxic tumor infiltrating lymphocytes (TIL), particularly Th1 cells, CD8 T cells, and NK cells is associated with increased survival of cancer patients. Such antitumor cellular immune responses can be greatly enhanced by adoptive transfer of activated type 1 lymphocytes.

Recently, adoptive cell therapy based on infusion of ex vivo expanded TILs has achieved substantial clinical success. Cytokine-induced killer (CIK) cells are a heterogeneous population of effector CD8 T cells with diverse TCR specificities, possessing non-MHC-restricted cytolytic activities against tumor cells. Preclinical studies of CIK cells in murine tumor models demonstrate significant antitumor effects against a number of hematopoietic and solid tumors. Clinical studies have confirmed benefit and safety of CIK cell-based therapy for patients with comparable malignancies.

Enhancing the potency and specificity of CIK therapy via immunological and genetic engineering approaches and identifying robust biomarkers of response will significantly improve this therapy.

The preparation and creation of CIK cells is done as described by Jakel et al:

*CIK cells are generated by culturing **peripheral blood lymphocytes (PBL)** with*

1. *interferon-γ (**INF-γ**) monoclonal*
2. ***antibody against CD3 (anti-CD3)** and*
3. ***IL-2** in a particular time schedule.*

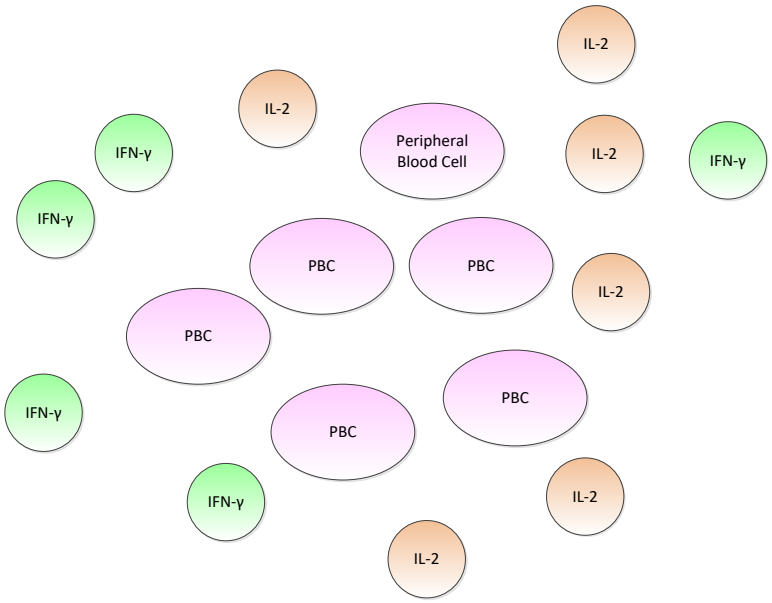
*The **cytokines INF-γ and IL-2 are crucial for the cytotoxicity** of the cells and anti-CD3 provides mitogenic signals to T cells for proliferation. Most of these CIK cells (87%) are positive for CD3 and for one of the T-cell coreceptor molecules CD4 (37.4%) or CD8 (64.2%), respectively.*

IFN-γ, added at day 0, activates monocytes providing crucial signals to T cells via interleukin-12 (IL-12) and CD58 (LFA-3) to expand CD56+ cells.

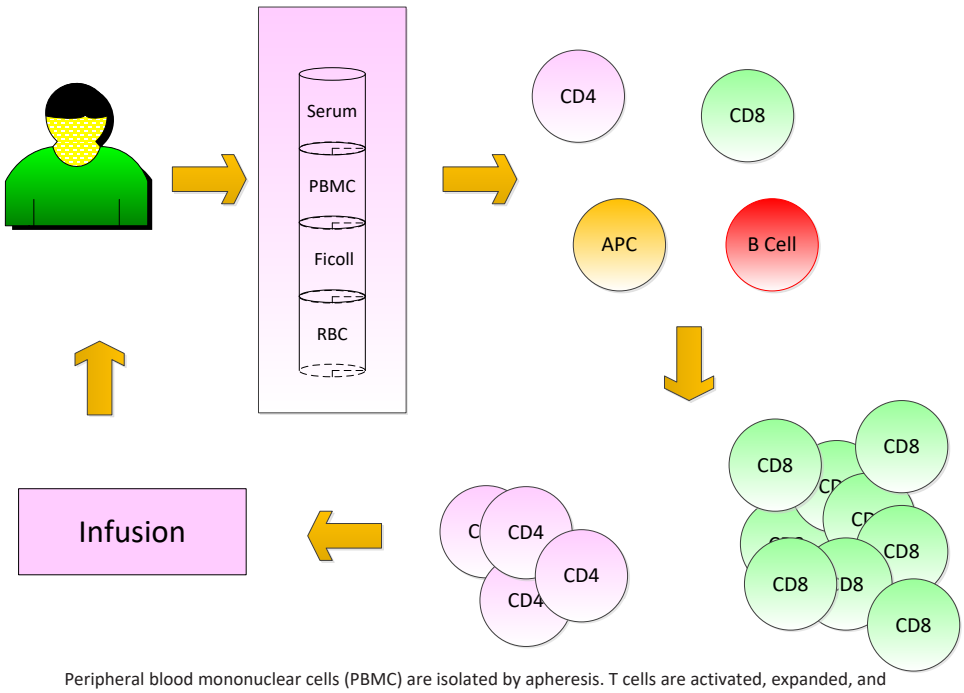
After 14 days of culture, 37.7% of cells are CD3+CD8+CD56+. These cells are referred to as natural killer T (NK-T) cells and represent the cell type with the greatest cytotoxicity in the CIK cell population.

Interestingly, these CD3+CD56+ double positive CD8+ T cells do not derive from the rare CD3+CD56+ cells in the starting culture but from proliferating CD3+CD8+CD56− T cells.

Their cytotoxicity is nonmajor histocompatibility complex (MHC)-restricted and they are able to lyse a variety of solid and hematologic tumors. Cell lysis is not mediated through FasL but through perforin release. CIK cell cytotoxicity depends on NKG2D recognition and signaling.



Jiang et al propose the following:



Peripheral blood mononuclear cells (PBMC) are isolated by apheresis. T cells are activated, expanded, and differentiated by anti-CD3 in the presence of cytokines including IFN-γ, IL-1α, and IL-2 for 14 to 21 days. These T cells, commonly called CIK, are then infused into patients. Jiang et al

Jiang et al prepare their cells as follows:

CIK cells have been evaluated as an adoptive cell immunotherapy for cancer patients in a number of clinical trials.

Peripheral blood mononuclear cells (PBMC) were isolated by apheresis.

T cells were then activated, expanded, and differentiated by

1. ***anti-CD3*** *in the presence of cytokines including*
2. ***IFN-γ,***
3. ***IL-1α, and***
4. ***IL-2***

*for **14 to 21** days to generate CIK, which were subsequently infused into patients.*

There are no significant clinical results for this in MDS but there are many Trials underway. One could suppose that this is a substantial third step after a BMT procedure. Logically it could be curative.

11.7 Observations

We now want to make some general and specific observations. WE shall discuss each as a separate topic.

11.7.1 Complexities of Epigenetics

Epigenetics has become as significant a factor in cancer as the pathway and immunological approaches. The impact of miRNA, lncRNA, methylation, acetylation, and other epigenetic elements are now understood as causative. However, the drivers initiating many of these are not clearly understood. The methylation in MDS for example is understood as a cause but what leads to the methylation is still speculative. For example, in melanoma one could speculate that backscatter X-rays in full body airport scans provide just the driver for methylation if it is applied at the right time. However, that is also speculative and no studies have been done. It is speculated that excess radiation, excess CAT scans or radiation for cancers can cause the methylation seen in MDS. Proof is lacking however.

11.7.2 Downside of Methylation

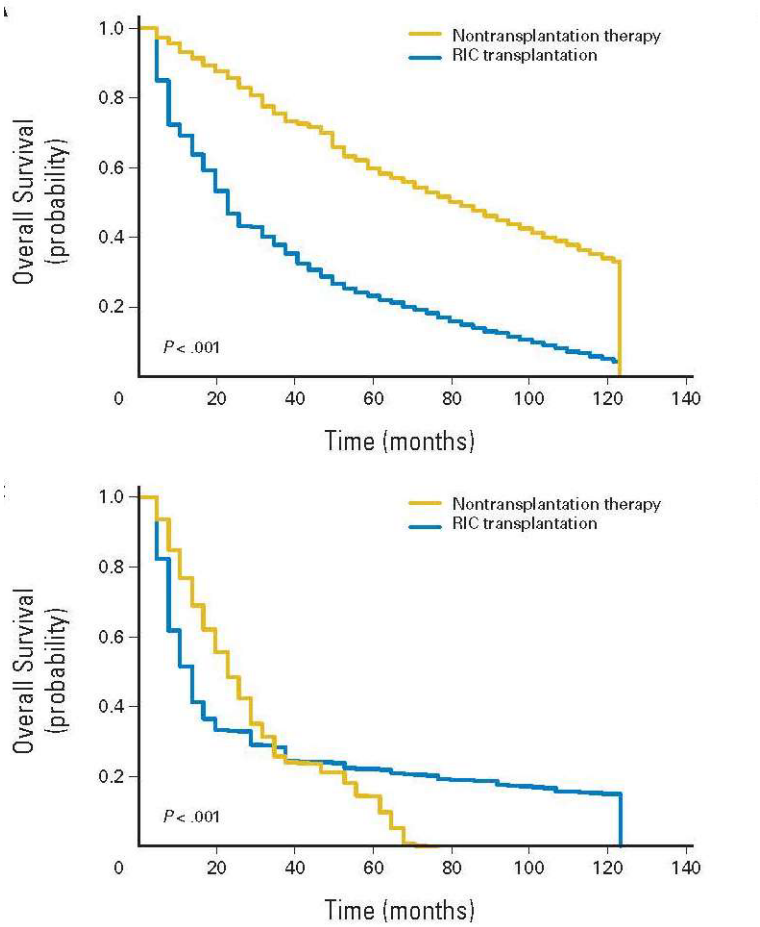
Methylation treatment with DNMT suppressors is known to drive down the blast percentage. However, it is a broad based therapeutic and demethylates many other cells. This may also give rise to secondary neoplasia, by activating proliferation genes in other cells in the body. It is not known how significant this is. It might result in sequelae as is found in Hodgkin's lymphoma but the sequelae there are often found 20-30 years later. Thus, since MDS occurs at 70 years of age that well exceeds any life expectancy.

11.7.3 Hypo vs Hyper Methylation

The problem with MDS is known to be hypermethylation. But there are many cases of hypomethylation as well. One then wonders if the approach taken herein applies to those cases as well.

11.7.4 Efficacy; Remission or Cure

Limited survival data is clinically available using the CIK approach. Koreth et al present data based upon a Markov model but we have considerable concerns about the approach. The results are shown below.



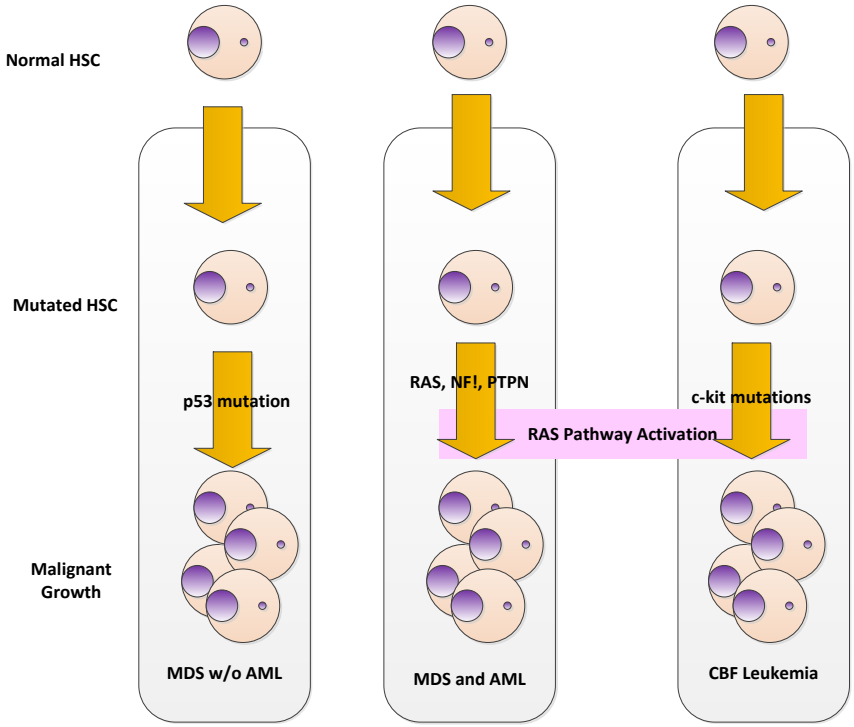
It should be noted that the top graph is for low to low intermediate and the bottom graph is high intermediate to high, using IPSS scoring. These Kaplan Meir curves show that for the high case we have a rapid drop and then a slow decline with about 20% at 10 years. Since the average age is about 70, the average life expectancy is 14 years and so 20% seem to have reached average life expectancy. In contrast the opposite is the case for the more indolent forms. The problem that we see is the initial conditions. Perhaps one would expect most patients have initial health conditions which would bias them against a BMT survival. Perhaps other health conditions are also a concern. The problem is that MDS is so complex and given the patients initial health status conditions it is expected that any case is different and Thus, any generalized result is problematic.

11.7.5 Final Observations

We end this section with some general observations regarding MDS, methylation and the use of transcription factor markers and their expression.

1. Is AML a definitive sequella to MDS: MDS is not a single disorder? There are multiple representations of the disease and there is no single clear representation. Many MDS patients end with AML and that is often the end state. AML can be severe and there are few treatments for it once it has commenced. In younger patients, a BMT is sometimes effective but the problem with those who start with MDS is that they are too old for BMTs.

From Niimi et al we have the following graphic which demonstrates the progression:



The normal progression of untreated MDS to AML is a common pathway and it does involve RAS activation. In addition, there are also p53 mutations in some MDS but not all.

2. What are the Causes of Methylation: We continue to struggle to understand what is the cause of the methylation that blocks the transcription factors and understanding that could be the most beneficial. Also, why the specific methylation targeting?

From Sonnet et al we have:

Hypermethylation and subsequent inactivation of genes are hallmarks of AML pathogenesis. Prominent examples include the epigenetically silenced tumor suppressor genes CDH1 or p15/CDKN2B. In addition, gene hypomethylation is frequently found in myeloid malignancies. The mechanistic link, however, between promoter hypomethylation and tumorigenesis is incompletely understood.

Global hypomethylation is common in many cancers, including AML, and is suspected to destabilize genome integrity by re-activating retrotransposons. Alterations in DNA methylation contribute to initiation, expansion, and evolution of the leukemic clone and promoter hypermethylation is a frequent observation in specimens of patients with MDS and AML. The mechanisms underlying the establishment of aberrant DNA methylation patterns are still largely unknown. Aberrant DNA methylation might be explained by the aberrant binding of transcription factors to their genomic target sequences.

3. What role do miRNAs play, if any, in the expression of these transcription factors?

As stated in Mendelsohn et al (see p 429):

The miRNAs miR-143, miR-145, and miR-146a mapping at 5q33 are significantly reduced in bone marrow cells isolated from 5q− syndrome patients. miR-145 and miR-146a regulate the toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP) and tumor necrosis factor receptor–associated factor 6 (TRAF6). TIRAP is a regulator of TRAF6, an E3 ubiquitin ligase, required for nuclear factor κB (NFκB) activation. Collectively, these proteins regulate innate immunity. Haploinsufficiency of miR-145 and miR-146a resulted in increased TIRAP and TRAF6 expression and activity in hematopoietic stem/progenitor cells. Transplantation of haploinsufficient miRNA145/146a cells or TRAF6-overexpressing cells into recipient mice resulted in several hallmark MDS/5q− syndrome phenotypes, such as thrombocytosis, neutropenia, dysplastic megakaryopoiesis, and propensity to transform to AML. These findings link innate immunity to MDS pathogenesis.

4. General use of a DMNT1 blocker and have putative systemic effects. It may demethylate many other genes and even demethylate histones Thus, uncovering genes which have been closed off by histone methylation. Is it Thus, better long term to have lower doses for that reason as well?

5. Cytogenic Abnormalities are more classic means for identifying MDS and AML as well as assessing prognostic values.

From Hoffman et al (Table 59.8) we have a complex array of cytogenic abnormalities amongst MDS patients. The Table shows the frequency of each and the mean survival when the abnormality remains. The observation can be made that they are so disparate and complex that any attempt to examine MDS in a broad sense is difficult. This is compounded by the low incidence as well.

Anomaly	Frequency (%)	Median Survival (Months)
Good-Prognosis Cytogenetics		
del(9q), NC	0.4	NR
del(15q), NC	0.4	NR
t(15q), NC	0.4	NR
del(12p), NC	0.8	108.0
+21, NC	1.1	100.8
–Y, +1	0.4	84.6
del(5q), isolated	8.2	80.0
+21, or +1	0.8	80.0
del(5q), NC	10.7	77.2
del(20q), isolated	1.9	71.0
del(20q), NC	2.2	71.0
–X, NC	0.5	56.4
No (normal karyotype)	49.5	53.4
del(5q), +1	2.5	47.0
+8, or +1	1.2	44.0
–Y, NC	2.7	39.0
–Y, sole	3.5	36.0
+1/+1q, NC	0.4	34.7
t(1q), NC	0.6	34.7
t(7q), NC	0.6	34.7
t(11q), NC	0.5	32.1
−21, NC	0.5	32.0
Intermediate-Prognosis Cytogenetics		
del(11q), NC	0.9	26.1
+8, NC	5.0	23.0
+8, isolated	3.8	22.0
t(11q23), NC	0.5	20.0
Rea 3q, NC	0.5	19.9
+19, NC	0.4	19.8
del(7q), isolated and NC	0.6	19.0
Any 3 abnormalities	2.8	17.1
del(11q), isolated	0.6	15.9
−7, +1	0.9	14.4
−5, NC	0.4	14.6
−7, sole	2.3	14.0
−7, NC	3.2	14.0
Poor-Prognosis Cytogenetics		
Complex, all	13.4	8.7
t(5q), NC	0.4	4/4
4-6 abnormalities	5.3	9.0
>6 abnormalities	3.9	5.0

6. Therapeutic Targeting: The approach taken in dealing with MDS is using the DNMT1 suppression drugs. These drugs have a broad spectrum use and frankly may have less than long term beneficial effects. However, when we see specific links being broken, then for example can we replace the genes not being expressed in the methylated cells? That would mean a targeting of specific proteins and more importantly an understanding of the proteins which have failed in terms of expression.

12 MELANOMA

There is an explosion of new cancer therapeutics. About ten years ago, we saw imatinib for CML and now we have quite a few for metastatic melanoma, once a terminal disease for certain. In the Melanoma case, we see some 20% may survive for extended periods of time. However, the average life extension may be only 6 months at a cost that may exceed $100K. In addition, there may need be several of the specific therapeutics used at one time.

A former Administration Health Care adviser has written on this of late[55]:

Many cancer patients, after getting a diagnosis of a terrifying disease, pursue any potentially promising therapy, regardless of the price. But the main cost driver is the fee-for-service payment system. The more doctors do for patients, the more reimbursement they receive. Surgeons earn more for every procedure. Oncologists typically make more money if they use newly approved drugs and the latest radiation treatments than if they use cheaper, older alternatives that work just as well. (This is because they get paid back the cost of the drug, in addition to an extra 6 percent of that cost — the more expensive the drug, the higher the compensation.)

His point is the 6% on the $100K charge. That is $6K per patient per six months. Take melanoma. The incidence is about 75K per year. Of that some 12K to 20K it the drug profile. At say $100K per person and assuming all persons, 20K, we would have in any one year $2B in costs and $120M paid to Oncologists. Is that too much? I guess it depends if you are in the 20% or the 80%.

He suggests changes:

First, over the next few years, the payment system needs to move away from fee-for-service toward a system of bundled payments, in which doctors are paid one fee for all the treatments involved in caring for a cancer patient.

This is a point well taken. But the problem is the way we compensate people based upon past assumptions.

Second, insurers have to give physicians information about where they are spending money.

I would suggest the patient also be informed. Patients all too often assume that the costs of the medication are low. They have no idea what the costs are. Moreover, the basis for the costs should also be known. One must remember that the drug companies have gone through multi-Phase trials of hundreds of patients each at tens of thousands per patients just to the management of the Trial. Recall that the CROs, the Clinical Research Organizations, generate almost $30B in annual revenue just managing the Trials to comply with the FDA. That is not money in the pockets of the Pharmas.

[55] http://opinionator.blogs.nytimes.com/2013/03/23/a-plan-to-fix-cancer-care/

Third, any change in payment methods must be accompanied by rigorous quality monitoring to ensure that there is neither under- nor over-utilization of care.

Quality, now just what do we mean by that? This is what drove the character nuts in the Zen and the Art of Motorcycle Maintenance. Really, is it nothing more than what is in the eye of the beholder?

Fourth, we need more "high touch" oncology practices. In these practices, nurses manage common symptoms before they escalate to the point that they require visits to the emergency room...

Part of this is that the Oncologists are dealing with a mass amounts of new and different genetically targeted drugs which address pathways that they may have never been exposed to in Medical School. One melanoma drug leads to a new skin cancer, an unexpected effect.

Fifth, we need better incentives for research. Many expensive tests and treatments are introduced without evidence that they improve survival or reduce side effects, and with poor information about which patients should receive them.

Here I would disagree. The Trials are somewhat extensive but when you apply something used over 600 people to 20,000 you get a whole new set of issues. A drug may have to be withdrawn.

One key question is who should receive the new therapeutics? How do we manage them? Cancer is terrifying to the patient. But we now have an environment where people can find out about these new medications and demand them. Physicians are then pressed to use them, albeit with little significant survival benefit, on average. Yet that 20% who do survive contain valuable information for the next step.

Thus, do we view use of the new therapeutics as the cost of continuing research or the cost of providing care?

12.1.1 What is Cancer

Let us begin by recalling the specific characteristics of what cancer is. As Hanahan and Weinberg state:

The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include

1. *sustaining proliferative signaling,*
2. *evading growth suppressors,*
3. *resisting cell death,*

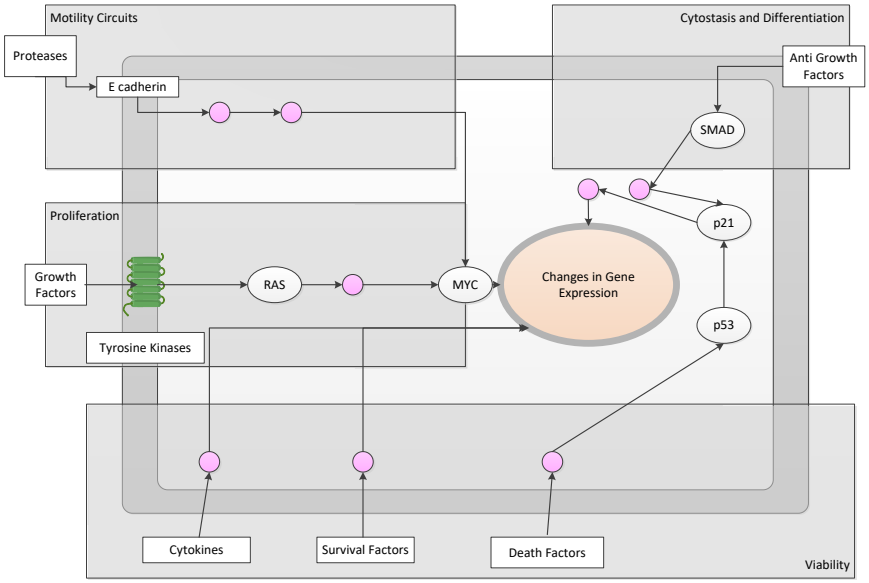
4. *enabling replicative immortality,*

5. *inducing angiogenesis, and*

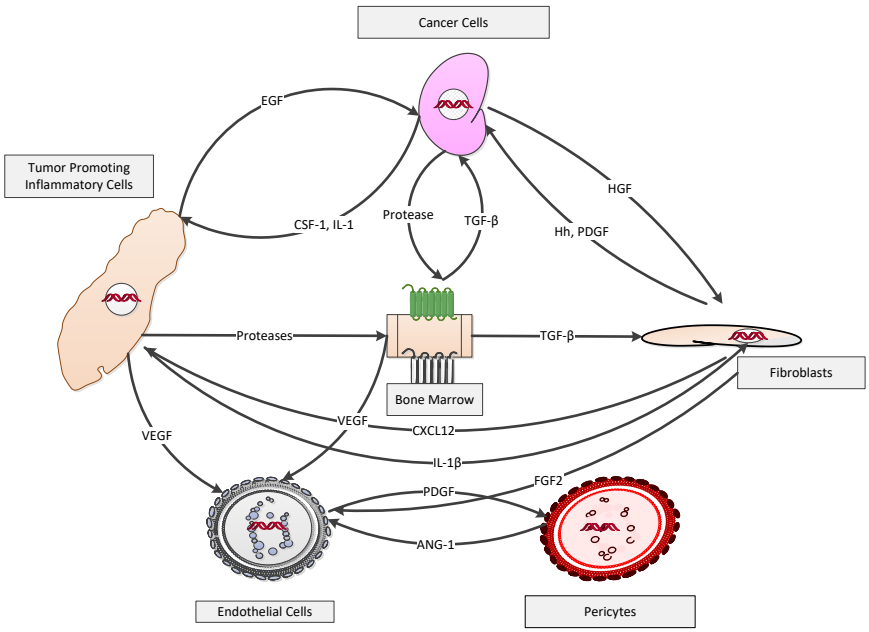
6. *Activating invasion and metastasis.*

Underlying these hallmarks is genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions.

The Figure below is a depiction of these processes based upon the above-mentioned paper. Note that this is a view of cancer from within the cell. The cell has the four characteristics shown: (i) motility, namely cancer cells move about such a melanocytes in melanoma in situ, where they move from the basal layer, (ii) proliferation, where they have activate mitotic behavior, as shown in the RAS-MEK pathway, (iii) differentiation, whereby the cell loses its functionality and becomes a nonfunctioning malignant cell, and (iv) loss of apoptosis, the cell essentially becomes immortal.



These characteristics map well onto the Hanahan-Weinberg terms. Cancer cells take on a life of their own. Therapeutics can then either attack them on the basis of the change in functionality or attack them outright. Classic chemotherapy used a meat cleaver approach, attacking any and all proliferating cells, including for example hair cells.



Thus the above depicts a somewhat global interrelationship between the cancer cell and the other cells within its environment. One of the key observations is that the cancer cells can also often take advantage of the surrounding cells and enlist them in the malignant cells own care and keep.

We are currently focusing on melanoma and from NCI we have the following estimated new cases and deaths from melanoma in the United States in 2013[56]:

- New cases: 76,690.
- Deaths: 9,480.

Furthermore the current therapeutics available, as described by NCI, are given as follows[57]:

Some melanomas that have spread to regional lymph nodes may be curable with wide local excision of the primary tumor and removal of the involved regional lymph nodes. A completed, multicenter, phase III randomized trial of patients with high-risk primary limb melanoma did not show a benefit from isolated limb perfusion with melphalan in regard to disease-free survival (DFS) or overall survival (OS) when compared to surgery alone.

Systemic treatment with high dose and pegylated interferon alpha-2b are approved for the adjuvant treatment of patients who have undergone a complete surgical resection but are considered to be at high risk for relapse. Prospective, randomized, controlled trials with both agents have shown an increase in relapse-free survival (RFS) but not OS when compared with observation.

[56] http://www.cancer.gov/cancertopics/pdq/treatment/melanoma/HealthProfessional

[57] http://www.cancer.gov/cancertopics/pdq/treatment/melanoma/HealthProfessional/page4

Clinicians should be aware that high-dose and pegylated interferon regimens have substantial side effects, and patients should be monitored closely. Adjuvant therapy with lower doses of interferon have not been consistently shown to have an impact on either RFS or OS.

Although melanoma that has spread to distant sites is rarely curable, both ipilimumab and vemurafenib have demonstrated an improvement in progression-free survival (PFS) and OS in international, multicenter, randomized trials in patients with unresectable or advanced disease, resulting in U.S. Food and Drug Administration (FDA) approval in 2011.

Vemurafenib is a selective BRAF V600E kinase inhibitor, and its indication is limited to patients with a demonstrated BRAF V600E mutation by an FDA-approved test.

Interleukin-2 (IL-2) was approved by the FDA in 1998 on the basis of durable complete response (CR) rates in a minority of patients (0% –8%) with previously treated metastatic melanoma in eight phase I and II studies. No improvement in OS has been demonstrated in randomized trials.

Dacarbazine (DTIC) was approved in 1970 based on overall response rates. Phase III trials indicate an overall response rate of 10% to 20%, with rare CRs observed. An impact on OS has not been demonstrated in randomized trials.

Temozolomide, an oral alkylating agent, appeared to be similar to DTIC (intravenous administration) in a randomized phase III trial with a primary endpoint of OS; however, the trial was designed for superiority, and the sample size was inadequate to prove equivalency.

Thus there are now a significant number of options for treating melanoma, classic ones using alkylating agents and interferon or Interleukin-2, and more recent one based upon an understanding of pathways and the details of the immune system.

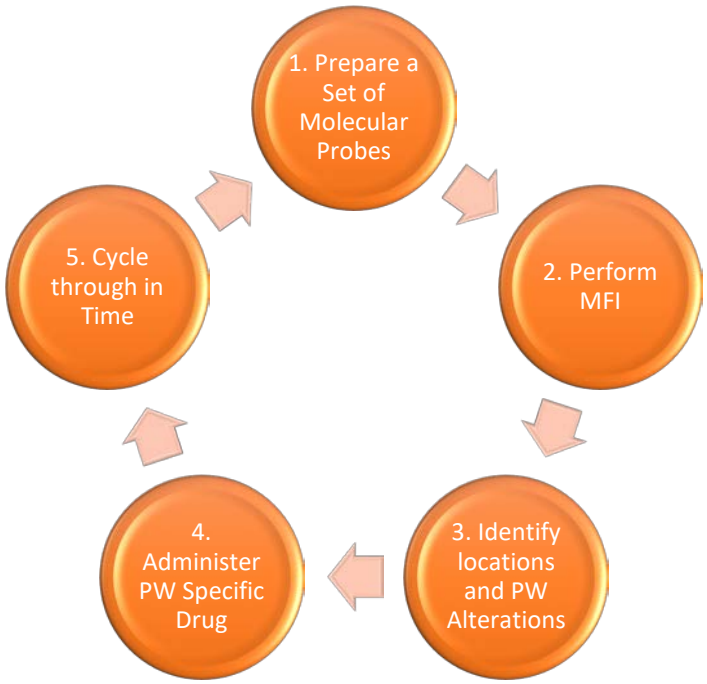
12.1.2 A New Therapeutic Paradigm

The classic therapeutic paradigm was to treat the disease systemically, namely suppress proliferation of cells, everywhere. Thus with something like methotrexate the cells stopped everywhere, including hair, and thus the patient went through an exhaustive and debilitating process. It also often times resulted in at best a suppression of the tumor for a short while and then a recurrence and death. With melanoma metastasis the process was often futile. Even with the early immune system approaches using interleukin and interferon, the side effects were present and the approach was for the most part systemic.

In the past decade with the understanding of pathways, in understanding the details of the immune system, and in being able to design targeted approaches to treatment we have now therapeutics that target the melanoma cells and not the entire system, almost.

The introduction of the use such as imatinib, a kinase inhibitor, for CML was a door opening step to dealing with cancers as genetically altered cells. Imatinib works to a degree, and when it fails another pathway element must be deployed.

We argue herein that there will be a new paradigm, which we depict below. It will be a paradigm based upon an understanding of cellular dynamics targeted at specific cells.



Namely:

1. Prepare a set of molecular probes which can tag the breakdown of specific pathway elements know to be specific to the metastasized cells. BRAF is but one example, and PTEN, cMyc, p53, are just a few others.

2. Perform a molecular functional imaging of the patient. This then allows for an identification of the location of the lesions, an assessment of the metabolic activity, and a clear indication of the gene expression aberrations in the tumor load.

3. Identify the specific localized PW aberrations and locations.

4. Prepare and administer therapeutics designed specifically to counter these aberrations.

5. Monitor patient and reiterate on periodic basis.

This is but one of the approaches, but it is an approach based on detailed understanding of the underlying malignancy at the gene level.

12.1.3 Approaches for Therapeutics

The key question is; how does one develop a therapeutic for melanoma, as an example? The answer as with many such questions is; it all depends. There are several approaches as suggested by the Hanahan and Weinberg updates. Let us summarize a few:

1. Pathway Modulators: The assumption in this class of therapeutics is that we understand the cancer at a pathway level and that there is some specific point or collection of points in the pathways which are malfunctioning. We assume we can identify that malfunction and then we further assume we can develop a therapeutic to modify the malfunction to align with the proper homeostasis of the cell.

2. Immunological Control: This approach uses the immune system but does so with specific emphasis on the uniqueness of the tumor cells. If we can identify specific cell surface markers that more accurate targeting by T cells may be achieved.

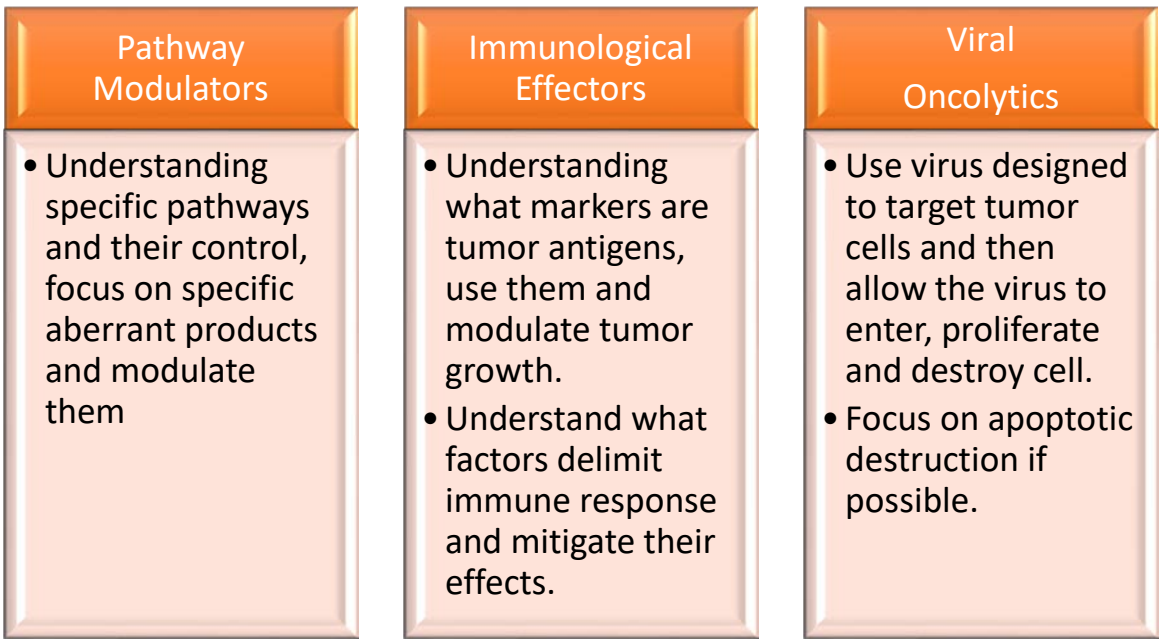
3. Oncolytic Viruses: This is a novel approach that again uses knowledge of specific cell surface markers. One can engineer viruses that attach only to malignant cells and then enter, multiply, and destroy the cells.

4. Extracellular Matrix Management: This is a more sophisticated approach using knowledge of the impact of the ECM on the cell.

5. Epigenetic Loss of Control: In this case we assume we are dealing with the genes in the pathways and that we have some epigenetic loss of control due to say miRNAs or methylation. Thus the therapeutic is one where we have a need to eliminate the methylation and thus reassert the gene expression or to likewise block the miRNA.

6. Gene Replacement: This approach assumes we have identified an aberrant gene, say resulting from some mutation or the like.

There are a set of putative therapeutic modalities for melanomas as well as cancers in general. We discuss them briefly here and detail them in the next section.

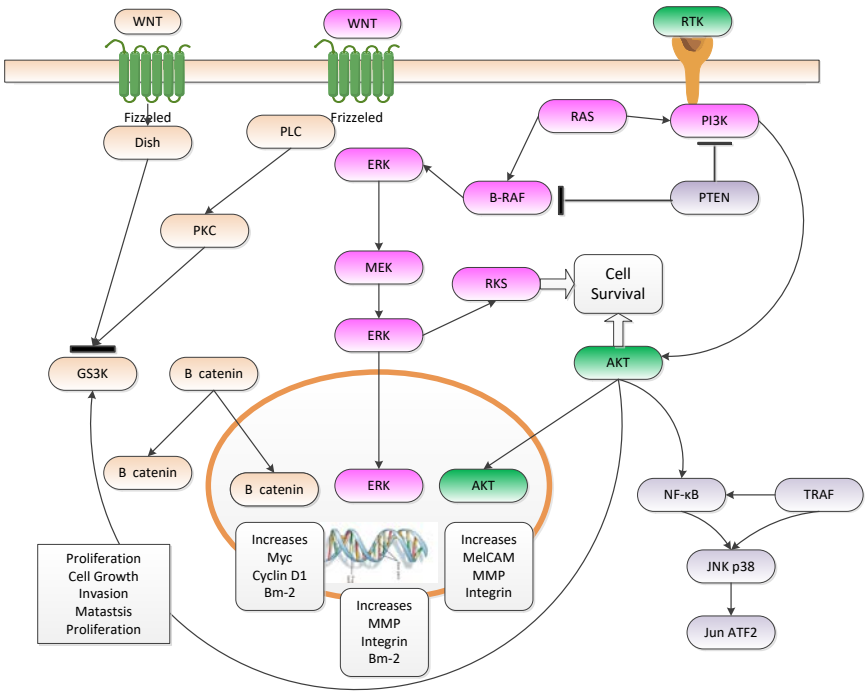


The above demonstrates the three directions we will focus on herein.

12.1.4 The Pathway Paradigm

The pathway paradigm is an articulation of our current understanding of pathways and how they can break down and result in excess proliferation, loss of functionality, movement, and all other characteristics of melanoma or cancer in general.

The following is an example of the classic pathway model as we now understand it.



Now pathways, as we have discussed in detail, control proliferation, movement, and functionality. The above is a graphic description of some of the genes related thereto.

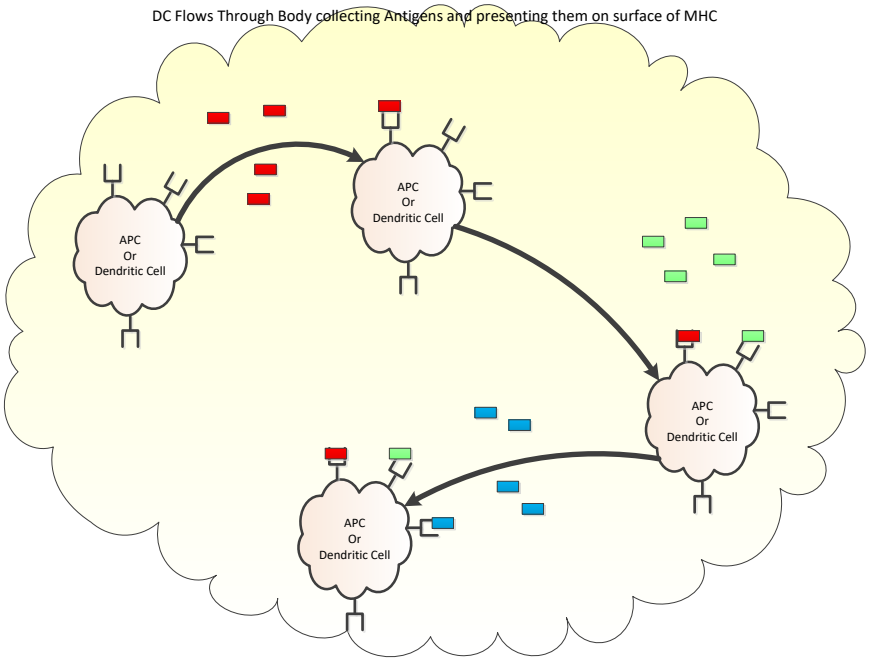
12.1.5 Immunological

Using the body's own immunological system as a way to attack tumors has been an attractive option for decades. Rosenberg had been approaching this in a systematic way since the late 1960s, and as understanding of the immune system has developed there have been improved options to affect such an approach. Simply stated, tumor cells often express surface markers which are antigens which the T cells can recognize and become activated. This is why we often see clusters of lymphocytes around tumor clusters. However the tumor cells have developed means and methods to block the T cell from becoming activated and thus resulting in the digestion of the cell. The tumor cells become protected from the normal action of the immune system.

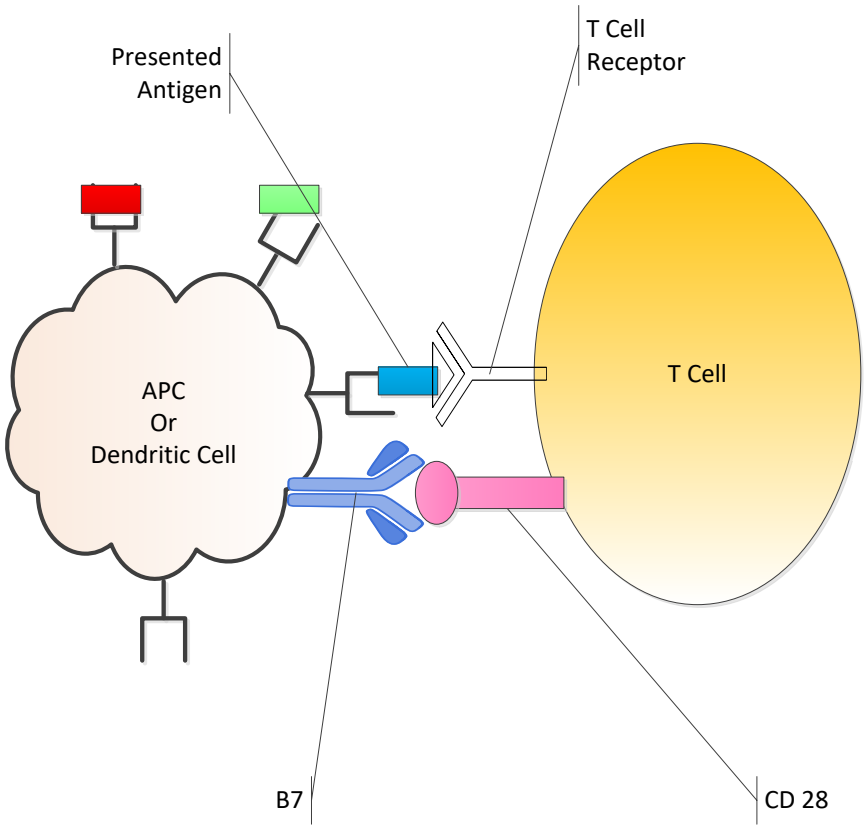
In melanoma the manner in which this happens is the use of a molecule the CTLA-4 which blocks a link normally attained with CD28 receptor. However by understanding this additional blockage one can then block the CTLA-4 from its blocking function and then allow normal operation of the immune T cell, namely destroying the melanoma cell.

We begin with a brief summary of how the immune system works in the case of some invasion. We assume a viral invasion but a malignant melanoma cell works the same, almost. We look at three steps.

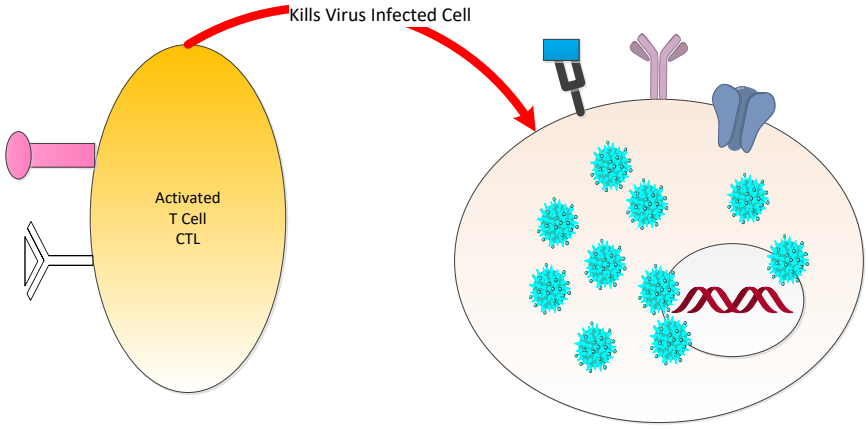
First an antigen presenting cell collects antigens as it floats around the body. Seen below it collects several different types to be presented to other immune system cells. The APC or Dendritic Cells are the sensors of invaders into the body.



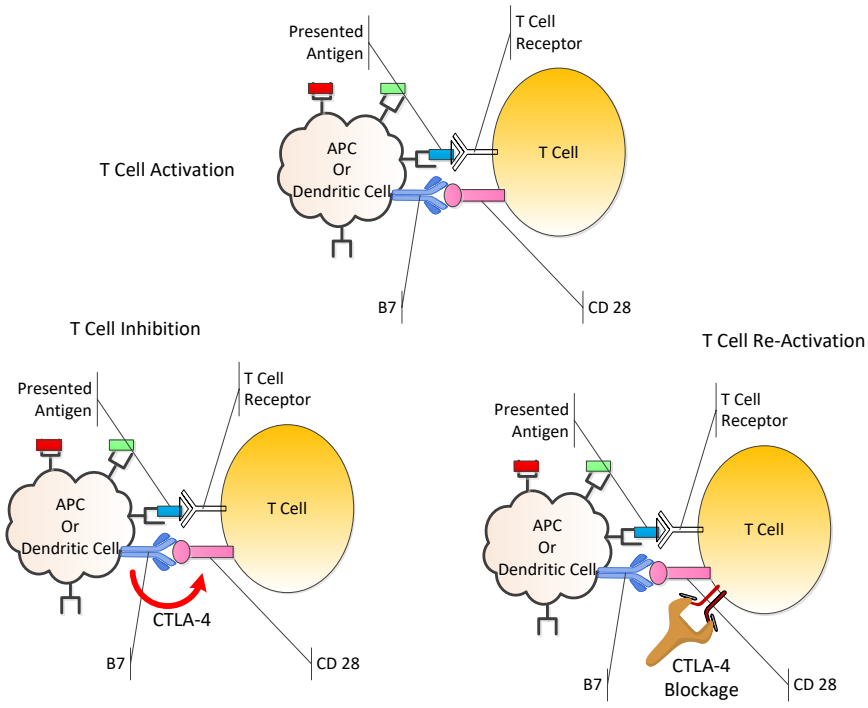
At a certain point the APC match up with a T cell and the antigen is then presented to the Tcell which in effect activates it to that specific “invader”



The activated T cell now can roam about activated for attaching to the invader. When such an invader, in this case a virus infected cell is seen, it attacks and destroys the cell.



Now we will apply this basic principle to the elimination of melanoma malignant cells. The Figure below depicts the three steps that are part of this process. First the APC sees the antigen. Now it is blocked by CTLA-4, which inhibits the destruction. Third, we find a molecule to block the attaching of the CTL-4 and thus reactivate the T cell. The 3 steps are shown below.



12.1.6 Therapeutic Action

We now use the basic principles above to describe how ipilimumab functions blocking CTLA-4.

We start by quoting Robert et al who state:

In summary, this trial showed that there was a significant improvement in overall survival among patients with previously untreated metastatic melanoma who received ipilimumab plus dacarbazine as compared with dacarbazine plus placebo. Adverse events other than those typically seen with dacarbazine or ipilimumab therapy were not identified. An increase in liver-function values is an important side effect that was observed more frequently than expected with the combination therapy.

Other ipilimumab-associated adverse events (enterocolitis and endocrinopathy) were observed, albeit at a rate that was lower than expected. The key side effects of ipilimumab were managed through adherence to treatment according to well established guidelines, including the administration of systemic glucocorticoids or other immunosuppressant agents.

Now we can examine the details of CTLA-4. As DeVita et al state:

CTLA-4 monoclonal antibody (ipilimumab) is a molecule expressed on lymphocytes that binds the B7-1 and B7-2 (CD80 and CD86) molecules on the surface of antigen-presenting cells. Engagement of the CTLA-4 molecule can suppress lymphocyte reactivity and interfere with IL-2 secretion and IL-2 receptor expression. The T-regulatory cells are the only lymphocytes in the resting circulation that constitutively expressed CTLA-4 on their surface; however, expression of CTLA-4 is transiently up-regulated after binding of the T-cell receptor. Multiple preclinical murine models have shown that CTLA-4 blockade can enhance immune-mediated tumor rejection when combined with vaccines.

Although the administration of anti–CTLA-4 monoclonal antibody to patients with metastatic melanoma has not been approved by the FDA as of the writing of this chapter, multiple clinical studies have shown that objective clinical responses can be achieved in patients treated with CTLA-4 blockade. In an updated study of 143 consecutive patients with metastatic melanoma treated with varying doses of anti–CTLA-4 either alone or in conjunction with peptide vaccination, an objective response rate of 17% was seen, including 10 patients (7%) with complete response. Substantial clinical experience with ipilimumab led to the observation that various unique patterns of clinical response could be observed in patients, including initial disease progression followed by tumor regression; mixed responses in which new lesions developed and subsequently stabilized or regressed; and late, slow continuous regression of metastatic disease.

The varied and delayed pattern of tumor response kinetics has been incorporated into strategies for clinical management of patients, for example, by observation of patients for 4-8 weeks beyond initial disease progression to detect late tumor responses. Preliminary data also indicate that a subset of patients achieving objective response or prolonged stable disease to an initial ipilimumab treatment course, who then subsequently demonstrate disease progression, can respond again to another treatment course of up to 4 doses.

*A multi-institutional prospective randomized trial was performed in 676 HLA-A*0201–positive patients with unresectable stage III or IV melanoma who received either (1) ipilimumab, (2) ipilimumab plus a gp100 peptide vaccine, or (3) the vaccine alone. Objective response rates were 11.0%, 5.7%, and 1.5%, respectively. Median overall survival was 10.1, 10.0, and 6.4*

months, respectively (P = .003 for ipilimumab compared with vaccine). There were 14 (2.1%) study drug–related deaths.

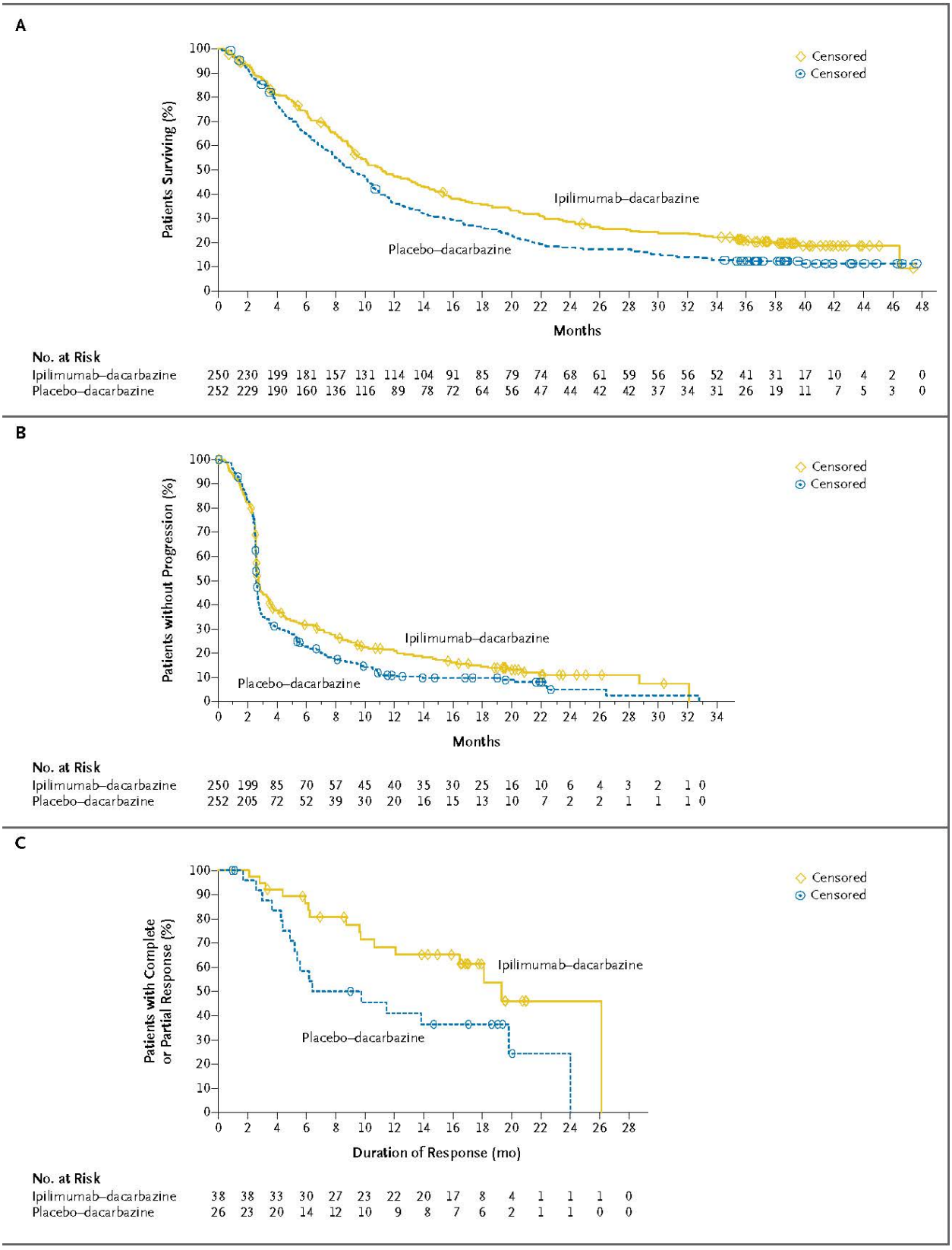
A second trial randomized 502 advanced melanoma patients without prior systemic treatment (except in the adjuvant setting) to dacarbazine (DTIC) every 3 weeks, up to 8 treatment cycles in combination with ipilimumab or placebo 10 mg/kg every 3 weeks up to 4 doses.

All patients without progressive disease or unacceptable toxicity were offered ipilimumab or placebo 10 mg/kg maintenance every 12 weeks. This trial also demonstrated improved median survival of 11.2 versus 9.1 months for patients receiving ipilimumab (P < .0009). For both randomized studies, 2- and 3- year survival estimates were approximately 10% greater for the ipilimumab containing arms compared to control.

Ipilimumab administration is associated with induction of inflammatory/autoimmune adverse events, including dermatitis; diarrhea/colitis/enteritis; and less commonly hepatitis and endocrinopathies, including hypophysitis, adrenal insufficiency and thyroiditis. Other rare autoimmune/inflammatory toxicity has been observed including nephritis, pneumonitis, uveitis, motor neuropathies, and immune-mediated thrombocytopenia. The colitis can rarely be associated with life-threatening bowel perforation.

Most of these side effects could be abrogated by the administration of steroids, although some patients may require additional immunosuppression for variable periods with anti-TNF agents. At the 3 mg/kg and 10 mg/kg dose levels of ipilimumab as a single-agent, about 15-20% and 25% of patients respectively may develop grade 3-4 autoimmune adverse events. The toxicity profile of ipilimumab may be influenced by concurrently administered agents; for example, in combination with DTIC, the expected rates of colitis/diarrhea were lower but rates of transaminase elevations were higher than expected for single-agent ipilimumab. In some phase 2 trials, a strong association was found between the probability of achieving an objective antitumor response and the development of some form of autoimmune adverse event.

The Figure below presents the Kaplan Meir curves for ipilimumab. Note that it extends survival for the 50% group to about 6 months. However there is a 20% who have indefinite survival. The question is what makes the 20% so unique and can we reproduce this.


A
Censored
Censored
Patients Surviving (%)
Ipilimumab–dacarbazine
Placebo–dacarbazine
Months
No. at Risk
Ipilimumab–dacarbazine 250 230 199 181 157 131 114 104 91 85 79 74 68 61 59 56 56 52 41 31 17 10 4 2 0
Placebo–dacarbazine 252 229 190 160 136 116 89 78 72 64 56 47 44 42 42 37 34 31 26 19 11 7 5 3 0
B
Censored
Censored
Patients without Progression (%)
Ipilimumab–dacarbazine
Placebo–dacarbazine
Months
No. at Risk
Ipilimumab–dacarbazine 250 199 85 70 57 45 40 35 30 25 16 10 6 4 3 2 1 0
Placebo–dacarbazine 252 205 72 52 39 30 20 16 15 13 10 7 2 2 1 1 1 0
C
Censored
Censored
Patients with Complete or Partial Response (%)
Ipilimumab–dacarbazine
Placebo–dacarbazine
Duration of Response (mo)
No. at Risk
Ipilimumab–dacarbazine 38 38 33 30 27 23 22 20 17 8 4 1 1 1 0
Placebo–dacarbazine 26 23 20 14 12 10 9 8 7 6 2 1 1 0 0

12.1.7 Pathway Managed

We have examined many of the pathways which when broken can lead to tumor cells and their proliferation. We will examine several of the therapeutic possibilities here and consider future directions for development.

We begin by reviewing some of the most recent developments in pathway based therapeutics for melanoma.

As Chapman et al state:

Vemurafenib is a potent inhibitor of mutated BRAF. It has marked antitumor effects against melanoma cell lines with the BRAF V600E mutation but not against cells with wild-type BRAF. A phase 1 trial established the maximum tolerated dose to be 960 mg twice daily and showed frequent tumor responses. A phase 2 trial involving patients who had received previous treatment for melanoma with the BRAF V600E mutation showed a confirmed response rate of 53%, with a median duration of response of 6.7 months. We conducted a randomized phase 3 trials to determine whether vemurafenib would prolong the rate of overall or progression-free survival, as compared with dacarbazine.

The mechanism of the induction of cutaneous neoplasia is under investigation, but it is speculated to involve the activating effect of vemurafenib on preneoplastic cells in which wild-type BRAF is further primed by upstream pathway activation. Several investigators have shown that vemurafenib and other inhibitors of RAF kinases can potentiate the activity of the MAPK pathway in cells with wild-type BRAF.

This finding might explain the favorable therapeutic index of vemurafenib in patients who have melanoma with the BRAF V600E mutation but also suggests is further primed by upstream pathway activation. Several investigators have shown that vemurafenib and other inhibitors of RAF kinases can potentiate the activity of the MAPK pathway in cells with wild-type BRAF.

This finding might explain the favorable therapeutic index of vemurafenib in patients who have melanoma with the BRAF V600E mutation but also suggests that vemurafenib could accelerate the growth of some tumors with wild-type BRAF. An important, related ongoing effort by many research groups is to clarify how melanomas become resistant to vemurafenib. Initial studies from several groups have indicated that the MAPK pathway is reactivated in resistant tumors. Although the precise mechanisms of reactivation are still being investigated, gatekeeper mutations in BRAF, which would prevent vemurafenib from binding BRAF, have not been observed. Our results show that single-agent vemurafenib improved the rates of response and of both progression- free and overall survival, as compared with dacarbazine, in patients with metastatic melanoma with the BRAF V600E mutation. These findings provide a solid foundation for the development of future combination therapies.

As Sosman et al state:

In conclusion, this trial shows a high rate of response to vemurafenib in patients with metastatic melanoma and activating BRAF mutations. These results independently confirm the high response rate and response duration shown in a phase 1 trial. The long follow-up period in our study provides critical information on long-term overall survival, not yet shown in the phase 3 trial comparing vemurafenib with dacarbazine.19 Targeted therapy aimed at oncogenic BRAF V600 induces responses in half the patients and a median survival of 16 months.

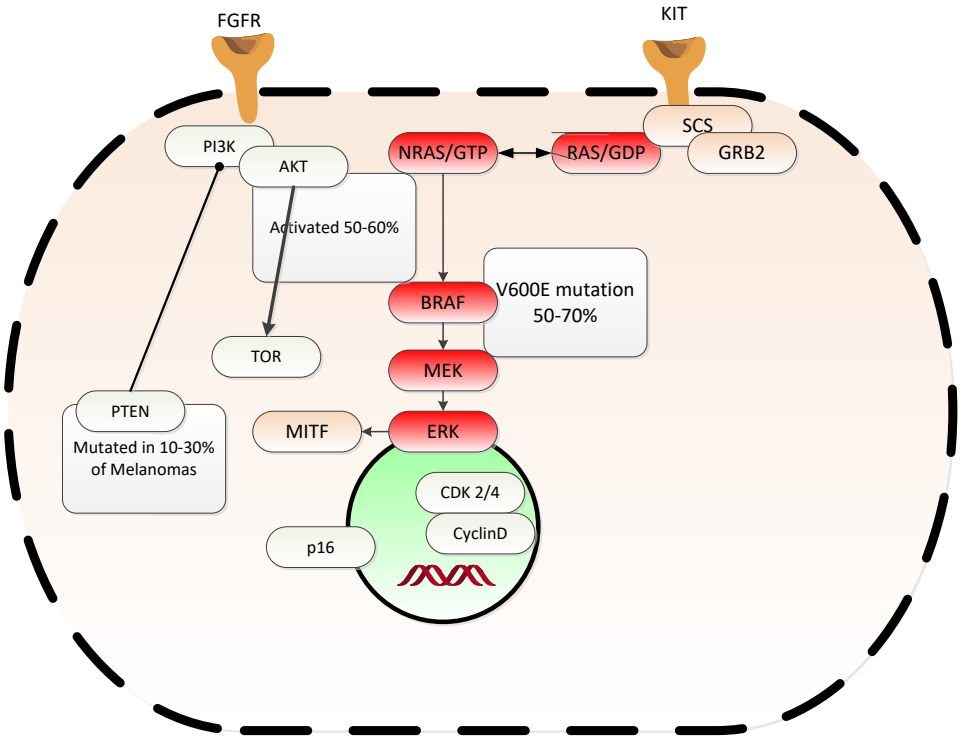
As Flaherty et al state:

Pharmacologic inhibition of the mitogen-activated protein kinase (MAPK) pathway has proved to be a major advance in the treatment of metastatic melanoma. The use of vemurafenib and dabrafenib, agents that block MAPK signaling in patients with melanoma and the BRAF V600E mutation, has been associated with prolonged survival and progression-free survival, respectively, in randomized phase 3 trials involving patients with previously untreated melanoma. Trametinib mediates blockade of MAPK kinase (MEK), which is downstream of BRAF in the MAPK pathway and has been associated with improved progression-free and overall survival in BRAF V600 melanoma (comprising both V600E and V600K mutations).

In spite of these advances, 50% of patients who are treated with BRAF or MEK inhibitors have disease progression within 6 to 7 months after the initiation of treatment. Several mechanisms mediating resistance to BRAF inhibitors through MAPK reactivation have been described, including the up-regulation of bypass pathways mediated by cancer Osaka thyroid kinase (COT), development of de novo NRAS or MEK mutations, and dimerization or variant splicing of mutant BRAF V600. In addition, MAPK-independent signaling through receptor tyrosine kinases, such as platelet derived growth factor receptor β, insulin-like growth factor 1 receptor, and hepatocyte growth factor receptor, have been associated with resistance. New therapeutic strategies are needed to address these resistance mechanisms.

Despite successful development of oncogene targeted therapy for chronic myeloid leukemia, gastrointestinal stromal tumor, and subtypes of breast cancer and non–small-cell lung cancer, it has not yet been possible to develop combination targeted therapies that circumvent acquired resistance. The combination regimen of BRAF– MEK inhibitors described here represents a successful attempt to combine targeted therapies in an oncogene-defined patient population. Furthermore, as a consequence of unique biochemical effects observed with BRAF inhibitors, this combination appears to be associated with a reduced incidence and severity of some of the toxic effects of monotherapy with either a BRAF or MEK inhibitor. We believe that the combination of dabrafenib and trametinib warrants further evaluation as a potential treatment for metastatic melanoma with BRAF V600 mutations and other cancers with these mutations.

Let us begin by considering some specific pathways, as relates to melanoma. We show below the B-RAF pathway with a V600 mutation.



All of the pathways shown above may be affected by mutations, suppression or over activation. We discuss here basically targets of opportunity.

12.1.8 Why BRAF?

Is BRAF the most critical pathway to target or is it a target of opportunity. More than likely it is both easier to target and 40-50% of melanomas have seen this mutation. It should be noted, however, than for the Irish, it is only 10%. Now we begin by summarizing from the report by Haq et al:

Activating mutations in BRAF are the most common genetic alterations in melanoma. Inhibition of BRAF by small molecules leads to cell-cycle arrest and apoptosis. We show here that BRAF inhibition also induces an oxidative phosphorylation gene program, mitochondrial biogenesis, and the increased expression of the mitochondrial master regulator, PGC1α. We further show that a target of BRAF, the melanocyte lineage factor MITF, directly regulates the expression of PGC1α. Melanomas with activation of the BRAF/MAPK pathway have suppressed levels of MITF and PGC1α and decreased oxidative metabolism. Conversely, treatment of BRAF-mutated melanomas with BRAF inhibitors renders them addicted to oxidative phosphorylation. Our data thus identify an adaptive metabolic program that limits the efficacy of BRAF inhibitors.

As reported by Science Daily[58]:

[58] http://www.sciencedaily.com/releases/2013/03/130308103416.htm

A multi-institutional study has revealed that BRAF-positive metastatic malignant melanomas develop resistance to treatment with drugs targeting the BRAF/MEK growth pathway through a major change in metabolism. The findings, which will be published in Cancer Cell and have been released online, suggest a strategy to improve the effectiveness of currently available targeted therapies.

"We were surprised to find that melanoma cells treated with the BRAF inhibitor vemurafenib dramatically change the way they produce energy to stay alive," says David E. Fisher, MD, PhD, chief of Dermatology at Massachusetts General Hospital (MGH) and a co-corresponding author of the Cancer Cell paper. "While current BRAF inhibitor treatment is a major improvement -- shrinking tumors in most patients and extending survival for several months -- patients eventually relapse. So there is an ongoing need to improve both the magnitude and durability of these responses."

In about half the cases of malignant melanoma -- the most deadly form of skin cancer -- tumor growth is driven by mutations in the BRAF gene. Research by investigators at the MGH Cancer Center and elsewhere has shown that treatment with drugs that block BRAF activity temporarily halts tumor growth. Combining a BRAF inhibitor with a drug that targets MEK, another protein in the same growth pathway, strengthens and extends the antitumor response. The current study was designed to investigate how BRAF inhibition changes metabolic activity within melanoma cells and to find other possible treatment targets.

The most common way that cells convert glucose into energy is called oxidative phosphorylation and largely relies on the activity of the cellular structures called mitochondria. Many cancer cells use an alternative mechanism that produces the energy compound ATP without involving mitochondria. A series of experiments by the MGH team revealed that the elevated BRAF activity in BRAF-positive melanoma cells suppresses oxidative phosphorylation by reducing expression of a transcription factor called MITF.

Suppressing production of MITF reduced levels of a protein called PGC1α that regulates the generation and function of mitochondria. But melanoma cells treated with a BRAF inhibitor showed elevated MITF activity, along with increased expression of oxidative phosphorylation genes and greater numbers of mitochondria. By switching to oxidative phosphorylation to supply the energy they need, the tumor cells increased their ability to survive in spite of BRAF inhibitor treatment.

"These findings suggest that combination treatment with mitochondrial inhibitors could improve the efficacy of BRAF inhibitors in malignant melanoma," says Fisher, the Wigglesworth Professor of Dermatology at Harvard Medical School. "Several small molecules that target mitochondrial metabolism have been identified by investigators here at the MGH and elsewhere, and laboratory investigations of specific combinations of BRAF inhibitors with mitochondrial antagonists are currently underway."

12.1.9 Oncolytic Viral Approach

Viruses function in a manner whereby they use the host cell resources to proliferate and then spread. A virus can recognize an appropriate cell in which it can activate its reproduction via a cell surface marker and then manage its way into the cell and then capture the cell for its own purposes. A simple HPV type wart is an example. In that case, we have the keratinocytes captured, and turned into a wart.

We now briefly consider the use of a virus to attack and kill a cancer. This has recently been reported by Rehman et al on the TVEC approach, a recently approved by the FDA therapeutic for melanoma. This is the reverse of the immune system killing viruses, it is the virus killing aberrant cells. As Rehman et al note:

Contemporary oncolytic virus therapy mediates tumor regression through two distinct mechanisms. First, many viruses possess an innate tropism for cancer cells where they can preferentially replicate and kill established tumor cells. Secondly, the dying tumor cells can serve as a target for cross priming tumor-specific immune responses generating systemic anti-tumor immunity. This second mechanism is important since tumor cells that are not infected by virus may nonetheless be targeted for elimination by the immune system. While most oncolytic viruses are given by direct injection into established tumors, several viruses can be delivered by the intravenous route avoiding the need for tumor localization and/or complex interventional administration strategies.

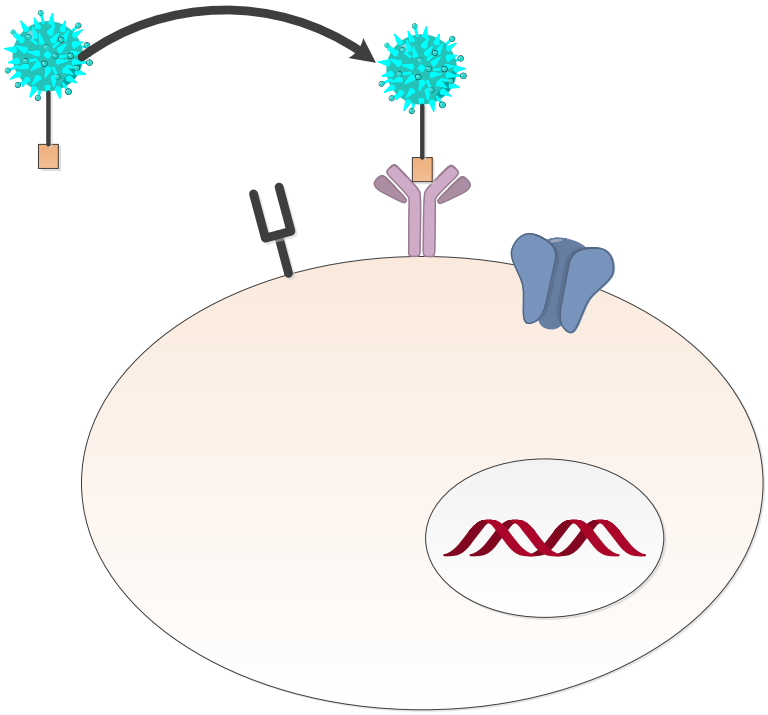
To date, the virus that has gained the most attention is an attenuated herpes simplex virus, type 1 (HSV-1) engineered to express human granulocyte-macrophage colony-stimulating factor (GM-CSF), termed Talimogene laherparepvec (T-VEC). This virus has been specifically adapted for selective tumor cell replication and induction of host immunity. The virus has now been tested in a prospective, randomized phase III clinical trial in which a significant improvement in durable and objective response rates were seen in patients with advanced melanoma. Based on the study results, T-VEC became the first oncolytic virus to achieve regulatory approval in the United States, Europe and Australia. The clinical implementation of oncolytic viruses is complicated by the need to properly store, prepare and administer the virus and the use of live, replicating viruses may not be familiar to many practicing oncologists. In addition, special attention of biosafety, infection control and potential close contact transmission of the virus demand additional education and training by healthcare providers.

We consider a simple three step process.

Step 1: Virus targets Specific Cell

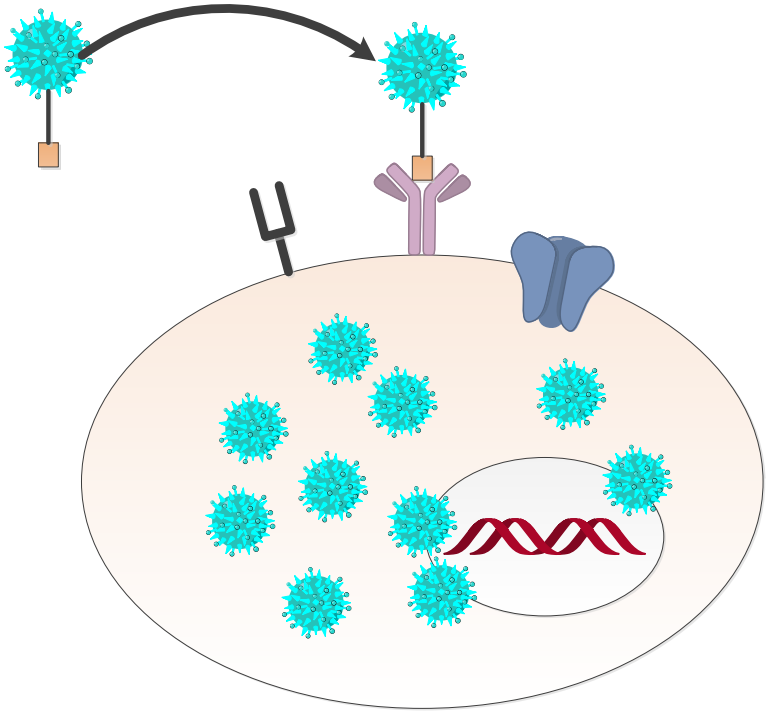
The figure below depicts an example of a virus which looks for a specific cell surface marker which it can then attach itself and enter the cell. For example HPV and HS-1 frequently attack specific epidermal cells and the generation of a wart is a classic example. The virus senses a specific cell type which it will use and then attaches and enters.

Now the problem with melanoma is that we have first to identify an appropriate cell surface marker unique to the melanoma cell and then engineer a virus to attack that specific cell. It becomes a targeted therapeutic.



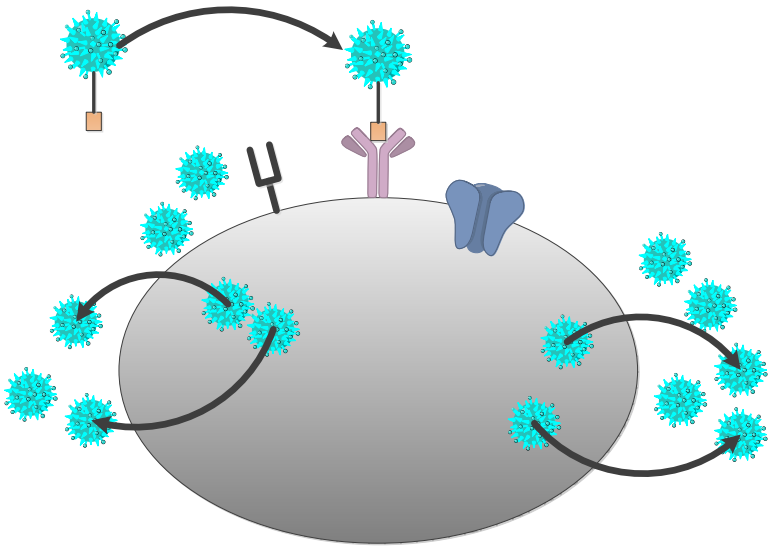
Step 2: Cell Enters and Proliferates

Viruses will then enter the cell and use the cells proteins to assist in its multiplication. In fact the cell becomes the host for this massive growth in the number of such cells.



Step 3: Virus Kills Cell

The final step is the killing of the cell by the explosive growth and expansion of the virus and then the virons go out and do the same with adjoining cells.



12.1.10Antigens and Cell Identification

The key to identifying a tumor cell is the antigen it presents. From Abbas and Lichtman we have the following typical antigens[59]:

[59] See Abbas and Lichtman p 392.

Type of Antigen	*Examples of Human Tumor Antigens*
Products of mutated oncogenes, tumor suppressor genes	Oncogene products: Ras mutations (~10% of human carcinomas), p210 product of Bcr/Abl rearrangements (CML) Tumor suppressor gene products: mutated p53 (present in ~50% of human tumors)
Unmutated but overexpressed products of oncogenes	HER2/Neu (breast and other carcinomas)
Mutated forms of cellular genes not involved in tumorigenesis	Various mutated proteins in melanomas recognized by CTLs
Products of genes that are silent in most normal tissues	Cancer/testis antigens expressed in melanomas and many carcinomas; normally expressed mainly in the testis and placenta
Normal proteins overexpressed in tumor cells	Tyrosinase, gp100, MART in melanomas (normally expressed in melanocytes)
Products of oncogenic viruses	Papillomavirus E6 and E7 proteins (cervical carcinomas) EBNA-1 protein of EBV (EBV-associated lymphomas, nasopharyngeal carcinoma)
Oncofetal antigens	Carcinoembryonic antigen on many tumors, also expressed in liver and other tissues during inflammation α-Fetoprotein
Glycolipids and glycoproteins	GM_2, GD_2 on melanomas
Differentiation antigens normally present in tissue of origin	Prostate-specific antigen in prostate carcinomas CD20 on B cell lymphomas

Now the Amgen announcement states:[60]

Amgen today announced top-line results from the Phase 3 trial in melanoma, which evaluated the efficacy and safety of talimogene laherparepvec for the treatment of unresected stage IIIB, IIIC or IV melanoma compared to treatment with subcutaneous granulocyte-macrophage colony-stimulating factor (GM-CSF).

The study met its primary endpoint of durable response rate (DRR), defined as the rate of complete or partial response lasting continuously for at least six months. A statistically significant difference was observed in DRR: 16 percent in the talimogene laherparepvec arm versus two percent in the GM-CSF arm.

The analysis of overall survival (OS), a key secondary endpoint of the study, is event driven. A pre-planned interim analysis conducted with the analysis of DRR has shown an OS trend in favor of talimogene laherparepvec as compared to GM-CSF.

[60] http://www.amgen.com/media/media_pr_detail.jsp?releaseID=1798143

"These are the first Phase 3 results of this novel approach to cancer therapy," said Sean E. Harper, M.D., executive vice president of Research and Development at Amgen. "A high unmet need exists in melanoma and we believe the innovative mechanism of action of talimogene laherparepvec may offer a promising approach for these patients."

The most frequent adverse events observed in this trial were fatigue, chills and pyrexia. The most common serious adverse events include disease progression, cellulitis and pyrexia.
Among the various types of skin cancer, melanoma is the most aggressive and also the most serious. Although melanoma accounts for less than five percent of skin cancer cases, or 132,000 cases globally each year, melanoma accounts for 75 percent of all skin cancer deaths.
Talimogene laherparepvec is an investigational oncolytic immunotherapy designed to work in two important and complementary ways - to cause local lytic destruction of tumors while also stimulating a systemic anti-tumor immune response.

We summarize this in the Table below.

Cytokine	Tumor Rejection in Animals	Clinical Trials	Toxicity
Interleukin-2	Yes	Melanoma, renal cancer, colon cancer; limited success (<15% response rate)	Vascular leak, shock, pulmonary edema
Interferon-α	No	Approved for melanoma, carcinoid tumors	Fever, fatigue
TNF	Only with local administration	Sarcoma, melanoma (isolated limb perfusion)	Septic shock syndrome
GM-CSF	No	In routine use to promote bone marrow recovery	Bone pain

12.1.11Others

There has been and will continue to be a growing number of therapeutics. The older ones, such as decarbazine, have been somewhat useful. Interferon, also, has been around for quite a while. Other chemotherapeutics have been tried but to no avail. The difference with the newer ones we have discussed herein is that they are based upon specific characteristics of the melanoma cell.

The trend appears to be several folds:

(i) Targeting specific pathway modifications, as they appear,

(ii) An endogenous approach utilizing the person’s own immune system as a targeting vehicle,

(iii) An exogenous approach using specific cell targeted viral probes.

We assume that many of the more broadly based approaches relating to cell proliferation modulation and angiogenesis modulation will continue to be explored but with greater knowledge of the cell dynamics the approaches discussed herein will be just as powerful if not more so.

12.1.12Classic Therapeutics

Typical classic therapeutics for the treatment of melanoma have been based upon the principles of blocking general cell proliferation. Twenty five to thirty years ago (see Fitzpatrick et al p 963, 1987) the recommended treatments were spotty at best. They used:

1. DTIC, dimethyl-triazeno-imi-diazole-carboxamide.

2. Nitrosoureas

3. Cis-platin, vinblastine, and DTIC or bleomycin

4. BCG with immunotherapy

Needless to say these had little effect, even though some survival stories were reported.

12.1.12.1 Alkylating Agents

Alkylating agents attack cells by binding to nucleophillic groups on cell constituents. They alkylate DNA and it is that process that is lethal to the cell. Alkylating agents function on proliferating and non-proliferating cells.

Decarbazine is one of the few alkylating agents used in melanoma. Several therapeutic efforts described herein use decarbazine as an adjunct. Alkylating agents become cytotoxic via a covalent bonding to nucleophillic groups on cell constituents. Decarbazine works through a metabolite not via its own properties directly.

Temozolomide is also an alkylating agent which requires a biotransformation akin to decarbazine. It also functions in a broad systemic manner and thus frequently has significant secondary effects.

12.1.12.2 Antimetabolites

Antimetabolites generally interfere with the availability of purine or pyrimidine nucleotide precursors. They may inhibit their synthesis or compete with them in DNA or RNA synthesis. At the present time they do not seem to be effective against melanoma. Methotrexate is a classic example of the antimetabolites.

12.1.12.3 Immune System Modulators

There has been an ever increasing interest in using the immune system to attack malignant cells, as intruders in the body. T cell responses and the related cytokines such as Interleukin 2 and Interferon have been an area of intense interest for well over twenty years. The early approaches as best exemplified in Rosenberg's book from 1992 describing his earliest observations and use of IL 2 and interferon.

12.1.12.4 Interleukin-2

Interleukin 2, IL-2, is a cytokine which is a driver in the proliferation, growth and differentiation of T cells. IL-2 induces the proliferation of antigen primed T cells as well as enhancing the natural killer cells, NK, for the attacking of the tumor cells.

From DeVita, Chapter 45, we have:

IL-2 was the first agent available for the treatment of metastatic cancer that functions solely through the activation of the immune system. Originally described as a growth factor for activated T cells, IL-2 was later found to exert multiple effects on cellular immune function and to induce tumor regression in mice. Subsequent clinical trials involving patients with renal cell carcinoma and malignant melanoma have demonstrated sufficient efficacy to establish IL-2 as an FDA-approved treatment for both of these malignancies.

In 1976, Morgan et al. demonstrated the existence of a growth factor present in the conditioned medium of lectin-stimulated human peripheral blood mononuclear cells that could sustain indefinitely the ex vivo proliferation of human T cells. This initial report was followed in short order by the isolation, biochemical characterization, and ultimately, the cloning of what was then termed the T-cell growth factor. Subsequently designated IL-2, this factor was shown to be a 15-kD polypeptide made up of 153 amino acids, the first 20 of which form a signal sequence that is proteolytically cleaved during secretion. Natural IL-2 is glycosylated, although the attachment of sugar moieties is not essential for biologic activity.

They continue:

IL-2 was administered at 600,000 to 720,000 IU/kg IV every 8 hours on days 1 to 5 and 15 to 19 of a treatment course. A maximum of 28 to 30 doses per course was administered; however, doses were frequently withheld because of excessive toxicity. Treatment courses were repeated at 8- to 12-week intervals in responding patients. During initial studies, patients underwent daily leukapheresis on days 8 to 12 during which large numbers of lymphocytes were obtained to be cultured in IL-2 for 3 or 4 days to generate LAK cells; these LAK cells were then reinfused into the patient during the second 5-day period of IL-2 administration.

This high-dose IL-2 regimen with or without LAK cells produced overall tumor responses in 15% to 20% of patients with metastatic melanoma or renal cell cancer in clinical trials conducted at either the NCI Surgery Branch or within the Cytokine Working Group (formerly the Extramural IL-2 and LAK Working Group).61 Complete responses were noted in 4% to 6% of patients with each disease and were frequently durable. Rare responses, usually partial and of shorter

duration, were also noted in patients with either Hodgkin's or non−Hodgkin's lymphoma, or non–small cell lung, colorectal, or ovarian carcinoma.

Randomized and sequential clinical trials comparing IL-2 plus LAK cells with high-dose IL-2 alone failed to show sufficient benefit for the addition of LAK cells to justify their continued use. Because of the quality and durability of tumor responses to this high-dose IL-2 regimen, IL-2 received FDA approval for the treatment of metastatic renal cell carcinoma in 1992 and for treatment of metastatic melanoma in 1998.

Long-term follow-up data for patients with melanoma and renal cell cancer treated in the initial high-dose bolus IL-2 trials presented to the FDA have confirmed the earlier findings of response durability, with median duration for complete responses yet to be reached and few, if any, relapses observed in patients free of disease for longer than 30 months. In fact, several patients have remained free of disease in excess of 20 years since initiating treatment. These data suggest that high-dose IL-2 treatment may actually have led to the cure of some patients with these advanced malignancies previously considered incurable.

The concept of a "cure" is thus achieved in a small group of patients using this modality. From DeVita, Chapter 19, we have:

The intravenous administration of high-dose IL-2 (aldesleukin) represents an effective treatment for patients with metastatic melanoma and the treatment most likely to provide long-term complete responses and cure in these patients.

IL-2 was first described as a T-cell growth factor in 1976. The DNA sequence of the gene coding for IL-2 was determined in 1983, and soon thereafter, the IL-2 gene was expressed in Escherichia coli, produced at high concentrations, and purified to homogeneity, and the biologic characteristics of this recombinant IL-2 were determined.261 Although early studies with IL-2 used material from mammalian sources, all clinical studies of IL-2 since 1985 have used the recombinant material.

The administration of IL-2 represented the first demonstration that purely immunotherapeutic maneuvers could mediate the regression of metastatic cancer. IL-2 has no direct effect on cancer cells, and all of its antitumor activity is a function of its ability to modulate immunologic responses in the host.

The FDA-approved regimen for the treatment of patients with metastatic melanoma using IL-2 involves the use of an intravenous bolus infusion of 600,000 to 720,000 IU/kg every 8 hours to tolerance using two cycles separated by approximately 10 days (maximum of 15 doses per cycle). Results of this treatment are evaluated at 2 months after the first dose, and if tumor is regressing or stable, a second course is then administered. This regimen was approved by the FDA for the treatment of patients with metastatic melanoma in January 1998 based on the ability of this IL-2 regimen to mediate durable responses.

The hallmark of IL-2 therapy is its ability to mediate durable complete responses in patients with widespread metastatic disease. In a report of the original 270 patients treated at 22 different

institutions that was the basis of the approval of IL-2 by the FDA, a 16% objective response rate was obtained, with 17 complete responses (6%) and 26 partial responses (10%).264 At the last full analysis of these 270 patients, the median duration of response for complete responders had not been reached but exceeded 59 months, and disease progression was not observed in any patient who responded for more than 30 months.

However IL-2 has been used for several years now and does have a positive effect. Yet it is still not curative in most cases. Perhaps the characteristics of ipilimumab blocking are necessary to fine tune the approach for specific melanoma metastatic cells.

12.1.12.5 Interferon

Interferon is a cytokine and work by interacting with the surface receptors of cells. There are three types of Interferon; α, θ, γ. Interferon enhances the activity of macrophages and NK cells and increases the expression of MHC molecules and it further enhances the production of IgG2b.

As Lartigue states[61]:

Interferon clearly act upstream of many important signaling pathways, and researchers have elucidated a plethora of different cellular roles besides their namesake activity of viral interference. For example, they play vital roles in regulating both the innate and adaptive immune responses, and in the activation, migration, differentiation, and survival of various different types of immune cell. In the 1990s, the role of IFNs began to be further delineated, and there was much excitement as it became apparent that they had so-called non-antiviral effects, a variety of effects on cell growth, apoptosis, and angiogenesis (new blood vessel formation) were observed, and this is when clinicians began to realize the potential anticancer applications of IFNs.

Over the decades that followed the discovery of the cytotoxic effects of IFNs, they were touted as a potential "magic bullet" treatment for cancer. While they ultimately did not offer the cure-all that many had hoped, they did become the first treatment for numerous types of hematological cancers and solid tumors, and offered significant hope to patients. At one time or another, they were used clinically and were standard-of-care treatment for chronic myeloid leukemia (CML), hairy cell leukemia (HCL), T- and B-cell lymphomas, melanomas, renal cell carcinomas, and AIDS-associated Kaposi sarcoma.

Thus interferons are a broad based therapeutic for many cancers and operate by exciting the immune system broadly. Yet as with any broad based therapeutic, especially one having such a strong influence on the immune system, it does have side effects. The author states:

A significant issue with type I IFN therapy is the substantial side effects experienced by patients, which include myelosuppression and nervous system disorders, and likely occur as a result of the broad cellular activity of this group of IFNs. The recently identified third type of IFNs, the IFNλs, activate similar downstream signaling pathways to the type I IFNs and have been shown

[61] http://www.onclive.com/publications/oncology-live/2013/march-2013/interferon-therapy-a-growing-family-feeds-new-interest-in-an-older-treatment/1

to share the same biological properties, including the antitumor activity. In fact, some studies suggest that IFNλ may have even more pronounced antiapoptotic and antiproliferative effects than IFNα. Since the lambda IFNs act through a unique receptor whose expression is limited to only certain cell types, it is possible that IFNλ could offer a less toxic therapeutic alternative for certain types of cancer. This is a hypothesis that is being heavily investigated.

As DeVita et al state:

Interferon alpha-2b was evaluated in three single-agent phase 2 trials in metastatic melanoma and was associated with a 22% objective response rate among 96 patients. No randomized trial comparing interferon-α with dacarbazine in metastatic disease has been conducted. On the basis of durable responses in some patients with metastatic disease, an adjuvant therapy trial was initiated in patients with high-risk stage 2 and stage 3 melanoma. Interferon-α was administered by intravenous infusion, 20 million U/m2, for 5 consecutive days every 7 days for 4 weeks during the "induction" phase. For a subsequent 48 weeks, 10 million U/m2 were administered by subcutaneous injection on alternate days for a total of three doses every 7 days in the "maintenance" phase.

The control arm was observation, the standard at the time that the trial was conducted. Two hundred eighty-seven patients were enrolled, 80% of whom had stage III melanoma; 20% had stage IIB melanoma. Pathologic staging was performed with regional lymph node dissection because sentinel lymph node biopsy had not yet been introduced. Overall survival was the primary end point, and the trial was designed to detect a 33% improvement.

Also from DeVita et al Chapter 19 we have:

Thus, interferon has been consistently shown to improve relapse-free survival compared to either observation or ganglioside GM2/keyhole-limpet hemocyanin vaccination. The longevity of this benefit has been established with 12.6 years of median follow-up … With twice the follow-up of the initial protocol-defined analysis, the improvement in relapse-free survival continued to be statistically significant (28% reduction in risk by hazard ratio; P = .02). However, with longer follow-up or by pooled analysis of E1684 and E1690, a definitive benefit with high-dose interferon in overall survival is lacking. With long-term follow-up …, high-dose interferon was associated with a statistically insignificant 18% improvement (P = .18).

The consistency of relapse-free survival data across all trials, in the absence of a consistent or durable survival benefit, has raised speculation that interferon may contribute to causes of death that are unrelated to melanoma recurrence, such as cardiovascular disease. In addition to the negative result for low-dose interferon in E1690, another phase 3 trial evaluated intermediate-dose interferon compared to observation in the adjuvant setting.

A total of 1,388 patients were randomly assigned to one of three arms: interferon 10 million units daily for 5 days out of 7 repeated for 4 weeks followed by 10 million units 3 times weekly for 1 year; interferon 10 million units daily for 5 days out of 7 repeated for 4 weeks followed by 5 million units 3 times weekly for 2 years; or observation. Neither interferon arm was associated with a significant improvement in the distant metastasis-free interval (7% improvement for

higher dose vs. observation; 3% improvement for lower dose vs. observation). Overall survival was slightly better for the higher-dose group (5% improvement compared to observation) but not different for the lower-dose group. As a consequence, such regimens remain investigational.

More importantly we have pegylated interferon, namely using polyethylene glycol, the "peg" term, coating to protect the therapeutic from degradation before being activated within the target cell, we have from DeVita et al:

Pegylation results in substantially slower clearance of interferon after administration. This allows for more stable drug exposure than can be achieved with the shorter-lived conventional interferon-α administered on alternating days by subcutaneous injection. To achieve a similar amount of drug exposure over the course of several days, pegylated interferon can be administered less frequently and at a lower dose per injection.

This results in a lower maximum concentration after each dose while increasing the percentage of the dosing interval for which interferon is at biologically active concentrations. Per month of therapy, this regimen is less toxic than the high-dose interferon regimen tested in E1684 and E1690. However, in EORTC 18991, 1,256 patients with resected stage III melanoma were randomized between observation and treatment with pegylated interferon 6 mcg/kg once weekly for 8 weeks by subcutaneous injection followed by maintenance at 3 mcg/kg weekly for 5 years. Given the long duration of therapy, it is not surprising that the cumulative toxicities reported were only marginally less than that observed with 1 year of high-dose therapy. Nonetheless, the dose intensity achieved during the induction phase was 88% of that intended, and for the maintenance phase it was 83%.

The primary end points of the trial were distant metastasis–free survival and relapse-free -survival. Patients treated with pegylated interferon had significantly reduced risk of relapse (18% improvement by hazard ratio; P = .01), but an insignificant improvement of distant metastasis–free survival (12% improvement; P = .11). Survival follow-up was immature at the time of the analysis of the primary end points, but no significant difference in -survival was observed.

Given the substantially improved tolerability of pegylated interferon, the data supporting an improvement in relapse-free survival is being reviewed by the FDA and European regulatory authorities. Three years of pegylated (100 mcg subcutaneously once weekly) was compared to 18 months of low-dose interferon (3 million units subcutaneously 3 times weekly) in a recently reported randomized trial among 898 patients with primary melanomas greater than 1.5 mm in thickness with or without microscopic involvement of regional lymph nodes.

The peg approach is but one of several where a transport vehicle such as peg or a nano particle is used to movement of the therapeutic[62].

[62] Again in the article by Lartigue it discusses such peg approaches as follows: Two pegylated IFNα agents, Pegasys and Pegintron, are approved for the treatment of chronic hepatitis B and C virus (HBV/HCV). The addition of several polyethylene glycol (PEG) molecules to IFNα helps to improve its pharmacokinetic and pharmacodynamic properties, shielding it from enzymatic degradation and increasing its half-life and stability. Since HBV and HCV infection is one of the leading causes of hepatocellular carcinoma (HCC), IFN treatment could help in the prevention

12.1.13Multiple Pathways

The BRAF inhibitors can be combined with MEK inhibitors to block the progression of squamous cell cancers. However, this is a dual pathway approach but for two malignancies. The question that should be posed is; can we identify sequential pathway changes which we can then block as they occur? For example, do we expect to see changes in PTEN, p53, cMyc and other pathway elements as the tumor progresses? If so, we have certain therapeutics which may be applied to block the proliferation effects of the loss of such pathway changes.

12.1.14Staged and Combined Therapeutics

With many cancers there has been substantial success with combined or staged use of therapeutics. Recent efforts with melanoma have tried the BRAF and immunological approaches combined with more classic approaches such as interferon. However, recent efforts to use the newer BRAF and immunological approach have met with some problems.

As Ribas et al state:

There has been great interest in testing combination therapy with the BRAF inhibitor vemurafenib and the cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4)–blocking antibody ipilimumab, currently the only two agents approved for the treatment of advanced melanoma on the basis of improved overall survival.1 Vemurafenib and ipilimumab have different mechanisms of action, and preclinical studies have suggested that BRAF inhibitors may enhance immune-cell function and antigen presentation.2-5 The only clinically significant overlapping toxic effects for these agents are in skin and liver, which rarely limit their use in patients. Therefore, ample rationale exists to investigate combined therapy with these two agents.

We conducted a phase 1 study of the concurrent administration of vemurafenib and ipilimumab. The primary goal was to evaluate safety and define an administration schedule for further clinical development. Patients were eligible to participate in the trial if they had metastatic melanoma with a BRAF V600 mutation and had not received previous therapy with a BRAF or MEK inhibitor or with CTLA-4 or programmed cell death protein 1 (PD-1)–blocking antibodies.

A second cohort of six patients was enrolled with the planned administration of a lower dose of vemurafenib (720 mg twice daily) together with the full dose of ipilimumab. Among the first four patients who were treated with this combination, elevations in aminotransferase levels (grade 3 in two patients and grade 2 in one patient) developed within 3 weeks after starting ipilimumab. After the toxic effects were reviewed, the remaining two patients in the second cohort received vemurafenib alone. In addition, two patients (one in each cohort) had elevations of grade 2 or 3 in the total bilirubin level with concomitant grade 3 elevations in aminotransferase levels…

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of many cases of HCC, researchers hypothesize. Indeed, IFN treatment, either alone or in combination with the purine analogue ribavirin, has been shown to decrease the incidence of HCC in patients with chronic HCV and HBV.

Thus the simple and direct step of staging and integrating may have less than beneficial secondary effects. There may be a simple logic for each approach but the combination may introduce yet as identified responses that are not worth the use.

12.1.15Observations

In the past few years there has been considerable success in designing and effecting therapeutics for metastatic melanoma.

We also have a set of choices. Consider the comments by Jang et al:

Patients with metastatic melanoma had few treatment options until 2011, when two drugs—ipilimumab and vemurafenib—were approved following advances in the understanding of melanoma biology and tumour immunology. Almost 50% of melanomas harbor mutations in BRAF, mainly at codon 600, which result in constitutive activation of the MAPK pathway.

The selective inhibitors of mutant BRAF Val600, vemurafenib and dabrafenib, showed major tumour responses, resulting in improved progression-free and overall survival in patients with metastatic disease, compared with chemotherapy. Antitumor activity was also recorded in brain metastases. The growth of cutaneous squamous-cell carcinomas is a unique side-effect of BRAF inhibitor therapy that is induced by the paradoxical activation of the MAPK pathway in cells with RAS mutations.

Trametinib, which targets MEK downstream of BRAF, also produced an overall survival benefit compared with chemotherapy, although tumour responses were less frequent than they were with BRAF inhibitors. Despite this robust antitumor activity, most responses to these drugs are partial and disease progression is typically seen at a median of 5—7 months. Multiple resistance mechanisms have been identified, including those that lead to reactivation of the MAPK pathway and other pathways, such as the PI3K-AKT-mTOR and VEGF pathways.

Some patients with BRAF Val600 mutant melanoma seem to also benefit from immunotherapies such as high-dose interleukin 2 and ipilimumab, which, by contrast with BRAF inhibitors, can produce durable complete responses. We review the available data to best guide initial treatment choice and the sequence of treatments for patients with BRAF Val600 mutant melanoma.

12.1.15.1 Extensions

This discovery leads to several observations of note:

1. One could have imagined something of this happening with Telomeres. It would almost be necessary to allow ongoing uncontrolled mitotic activity. Thus, despite the fact that there is no surprise here we do have a specific target, namely the activator of TERT.

2. Melanoma, as most other cancers, has a multiplicity of changes to genes. There are ligands, receptors, pathway elements, transcription factors, and the telomere issues as well. It is clear that

no single factor is the dominant one as of yet. BRAF as a target works for a while and then there is a work around. Thus cancer is an evolving process, and one which may be highly adaptive.

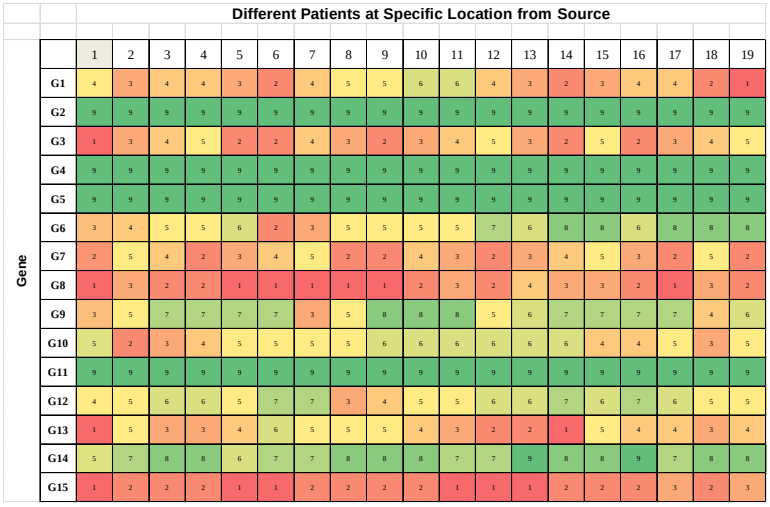
3. A Conjecture: As we have learned more and more as to aberrant genes and their products, as well as miRNAs, and their effects, one could envision several uses of malignancy profiling. We consider that in two steps.

Step 1: Profiling a Specific Patient at Various Locations. As shown below we consider a specific patient and then profile gene expression as a function of distance from the site of initiation, if such was possible. Then we can see how various aberrant genes are being expressed over the distances measure from the source. One would suspect that distance must be measured in some normalized manner but we leave that as an exercise for the student at this time. This gives us a profile for a specific patient, perhaps one for developing therapeutics.

Specific Patient at Different Locations From Source

Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
G1	7	6	6	5	5	5	5	4	4	4	3	3	3	2	2	2	1	1	1
G2	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G3	6	6	5	5	4	3	2	1	1	1	1	1	1	1	1	1	1	1	1
G4	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G5	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G6	6	6	6	5	5	4	4	3	3	2	2	1	1	1	1	1	1	1	1
G7	7	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
G8	7	4	4	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G9	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
G10	8	7	6	6	6	5	5	5	5	5	4	4	3	3	3	3	3	2	1
G11	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G12	5	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
G13	6	5	4	3	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G14	7	7	6	6	6	6	5	5	4	4	3	3	3	2	2	2	2	2	2
G15	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Step 2: The Same Location but across a Large Pool of Patients: Again we look now at the same distance from the source, perhaps at the same time, again an exercise for the student, and we get profiles of the expression of aberrant genes. This allows us to understand the between patient differences.



3. Is Seventy Enough? The study did an analysis on 70 lesions. Perhaps that is not enough. Furthermore based upon our previous comments perhaps a correlative study is demanded as well, by patient and by distance.

4. One of the problems I see is the continually hyping of the results as if this is finally the right answer. Anyone even slightly familiar with the field will understand that each input is vital but assembling them in a cohesive whole is essential. The systems approach is the sine qua non, but that cannot be done without the continual bench work required to understand the details.

For example in an article in the Boston Globe the reporter states[63]:

Now scientists working independently in Boston and Germany have made a surprising discovery: a set of genetic mutations found in most melanomas, the deadliest skin cancer. The presence of these mutations in the vast majority of tumors studied suggests that the researchers may have stumbled upon a fundamental mechanism involved in a hallmark trait of cancer cells—their ability to live forever—that could one day be targeted by drugs.

Outside researchers said the work, published online Thursday in the journal Science Express, is exciting because the conclusion is the opposite of what many exhaustive studies of cancers have shown.

In reality as we have discussed, it was imperative that the Telomeres be preserved in metastasis. Millions of rapid mitotic changes in a stem cell must survive and that means keeping Telomeres and that means lots of TERT. Somehow the conclusion was logical, consistent and not at all unexpected especially given what else has been found in the past decade.

The article continues:

[63] http://www.boston.com/news/science/blogs/science-in-mind/2013/01/24/boston-researchers-discover-mutations-that-underlie-melanoma-junk-dna/mNIYVavGfVsvstVj5eNfzO/blog.html

Both teams zeroed in on mutations in a part of the genome called a promoter, which acts like a volume knob on a stereo to control gene activity. The gene that the promoter controlled happened to be one that has long been of interest in cancer because it creates part of an enzyme called telomerase, which enables cancer cells to continue to divide indefinitely as one of its key jobs. Still, it wasn't easy for the researchers to convince themselves that what they found, underlying more than two-thirds of melanoma cases, was real.

One would expect this and if one looks at say the miRNA discoveries, they all add up to what controls the ultimate expression of mitotic survival.

5. Theraputics: Can we expect therapeutics from this understanding? Good question. Kinase inhibitors are now well understood, one could in theory build an inhibitor here as well. Is this the target, another target, necessary, helpful, we can only guess. Yet the above Conjecture may allow for the development of a therapeutic profiling plan for melanoma and other malignancies.

12.1.15.2 A System View of Therapeutics

We have developed models for cancer cell propagation and mutation. These models are based upon physical principles but clearly require experimental validation and verification. As one would expect, they are most likely first generation techniques, rough and requiring significant iterative modifications. However they are a paradigm for development.

They are also a paradigm for measuring progression and for determining what cellular modifications are where and when and thus being able to determine the best therapeutic practice.

In previous work we have developed a detailed temporal-spatial model for the propagation of cancer cells in a metastatic environment. We have also combined with that the effect of cellular mutations or equivalent expression changes, perhaps driven by epigenetic factors. We have further suggested that using molecular functional imaging that we could effectively profile the metastatic behavior consistent with the model. Having such a non-invasive data set, taken temporally over some period, could provide a powerful model for prognostic as well as putatively therapeutic usages.

Thus unlike the microarray approach, which is invasive, the molecular functional imaging, MFI, approach could provide a methodology that enable whole body assessment of the progression of the metastasis as well as the genetic alterations which are following the change.

13 PROSTATE CANCER

Prostate cancer, PCa, is a significant cancer especially in older men. It is more than likely a highly heterogeneous cancer and thus unlike many of the others we have examined there may not be a single strategy.

As Drake, has indicated:

Prostate cancer is not traditionally considered an immunologically responsive malignancy like melanoma or renal cell carcinoma, yet the prostate glands of men with cancer are frequently diffusely infiltrated with both CD4 and CD8 T cells, and several factors suggest that adenocarcinoma of the prostate might prove an attractive target for immunotherapy.

First among these is the slow-growing nature of the disease, allowing time for immunological intervention to overcome immunosuppressive factors1 in the tumor microenvironment and to mount a clinically meaningful response.

Second, serum PSA level, while not a true surrogate marker, is routinely utilized in clinical decision making, and can serve to guide the development of immunotherapy approaches.

Third, both proteomic and microarray analyses of prostate cancer progression have delineated a number of relatively tissue-specific proteins that may serve as tumor/tissue antigens.

Finally, abundant preclinical data suggest that an antitumor immune response can be elicited, particularly when active immunotherapy is combined with maneuvers to mitigate tolerance such as immune checkpoint blockade, androgen ablation, or radiotherapy.

At least five phase III immunotherapy trials have been initiated in the context of metastatic, castrate-resistant prostate cancer, but none have yet met their predetermined end points

13.1 THE PROSTATE

The prostate is a glandular organ which appears upon microscopic examination as a multiplicity of glands with muscle, nerve, blood, and other stromal and parenchymal tissues. It has a high incidence of cancer as men age and the cancers for the most part are indolent, namely have low chance of metastasis, yet a fraction show highly aggressive behavior. Also an alleged precursor of PCa, prostate cancer, is High Grade Prostate Intraepithelial Neoplasia, an inflammatory disorder wherein the existing glandular regions generally composed of basal and luminal cells, demonstrate significant growth within the gland itself. It has been argued that this is a natural precursor to PCa but we have demonstrated that the conclusion has significant exceptions. Yet we know that inflammation is a driver to cancers and there thus is a putative correlation but not a causation. (See Nunzio et al):

Evidence in the peer-reviewed literature suggested that chronic prostatic inflammation may be involved in the development and progression of chronic prostatic disease, such as BPH and PCa,

although there is still no evidence of a causal relation. Inflammation should be considered a new domain in basic and clinical research in patients with BPH and PCa.

PCa is quite complex on a genetic basis. Berger et al have discussed this at length. They state:

Prostate cancer is the second most common cause of male cancer deaths in the United States. Here we present the complete sequence of seven primary prostate cancers and their paired normal counterparts. Several tumors contained complex chains of balanced rearrangements that occurred within or adjacent to known cancer genes. Rearrangement breakpoints were enriched near open chromatin, androgen receptor and ERG DNA binding sites in the setting of the ETS gene fusion TMPRSS2-ERG, but inversely correlated with these regions in tumors lacking ETS fusions. This observation suggests a link between chromatin or transcriptional regulation and the genesis of genomic aberrations. Three tumors contained rearrangements that disrupted CADM2, and four harbored events disrupting either PTEN (unbalanced events), a prostate tumor suppressor, or MAGI2 (balanced events), a PTEN interacting protein not previously implicated in prostate tumorigenesis. Thus, genomic rearrangements may arise from transcriptional or chromatin aberrancies to engage prostate tumorigenic mechanisms.

We have further examined this in detail in McGarty, Prostate Cancer (2012). Now in 2009 Drake stated:

Prostate cancer is not traditionally considered an immunologically responsive malignancy like melanoma or renal cell carcinoma, yet the prostate glands of men with cancer are frequently diffusely infiltrated with both CD4 and CD8 T cells, and several factors suggest that adenocarcinoma of the prostate might prove an attractive target for immunotherapy.

First among these is the slow-growing nature of the disease, allowing time for immunological intervention to overcome immunosuppressive factors1 in the tumor microenvironment and to mount a clinically meaningful response.

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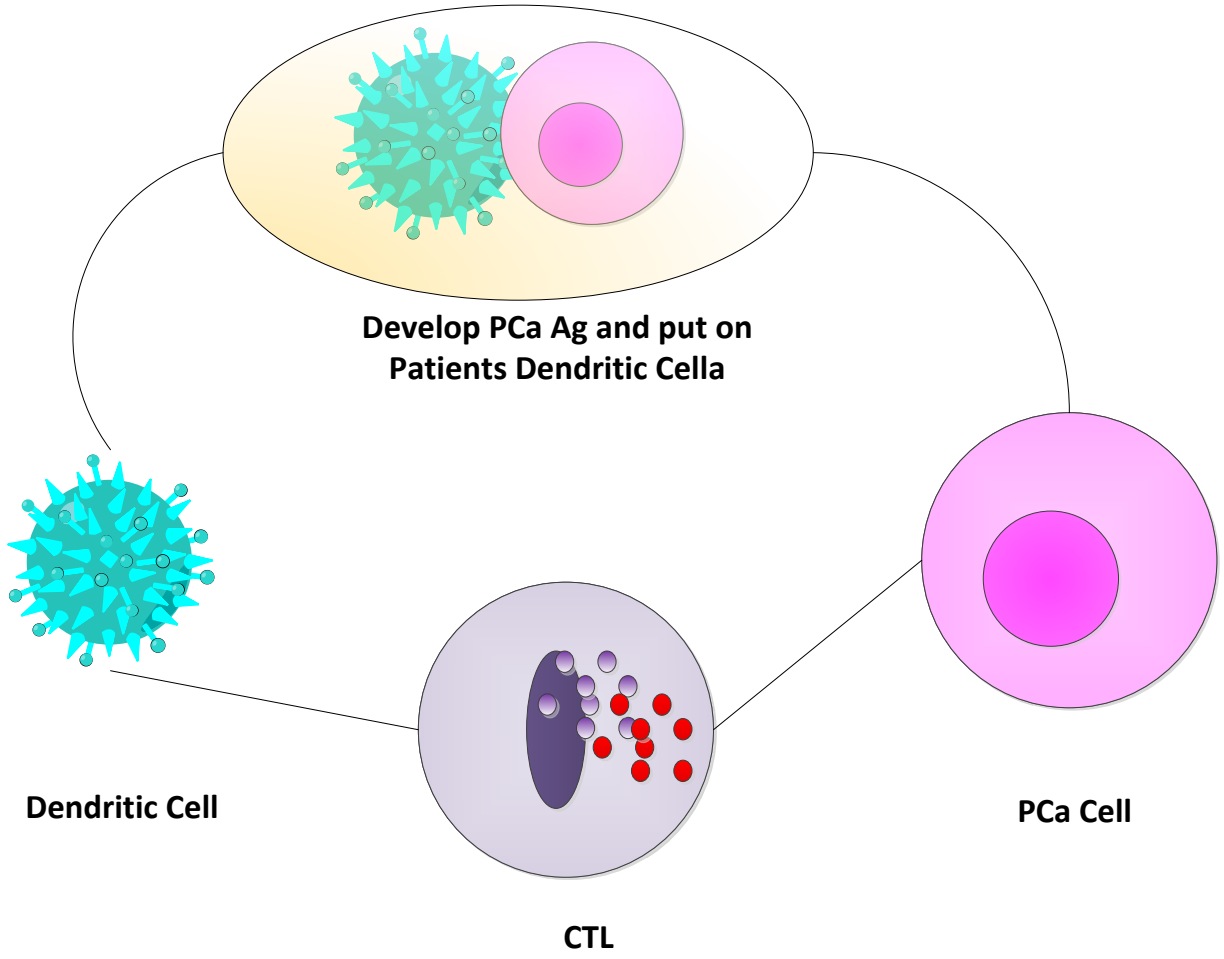
At least five phase III immunotherapy trials have been initiated in the context of metastatic, castrate-resistant prostate cancer, but none have yet met their predetermined end points

Drake was noting the potential for immunotherapeutic approaches for this solid tumor. It is well known that the prostate is subject to various inflammatory factors and that these factors have been linked to cancer changes. The counter would be to examine using the immune responses to

address the changes. As we noted in melanoma, when observed, a melanoma often has an accumulation of T cells, CTLs, indicating the natural defense mechanism.

13.2 Current Techniques; Dendritic

One of the earliest treatments of PCa using an immunotherapeutic approach is to use the patient's dendritic cells and prime them. Recall that the dendritic cells are out in the body searching for intruders. When they find one they then bring it back to the immune system for presenting and for activating the immune system. Thus, rather than modifying a T cell or an NK cell directly, the approach seeks to "prime" the dendritic cells which will then start the immune response. This is an example of examining the many entry points into using the immune system.



As Westdorp et al note:

Prostate cancer (PCa) is the most common cancer in men and the second most common cause of cancer-related death in men. In recent years, novel therapeutic options for PCa have been developed and studied extensively in clinical trials. Sipuleucel-T is the first cell-based immunotherapeutic vaccine for treatment of cancer. This vaccine consists of autologous mononuclear cells stimulated and loaded with an immunostimulatory fusion protein containing

the prostate tumor antigen prostate acid posphatase. The choice of antigen might be key for the efficiency of cell-based immunotherapy. Depending on the treatment strategy, target antigens should be immunogenic, abundantly expressed by tumor cells, and preferably functionally important for the tumor to prevent loss of antigen expression. Autoimmune responses have been reported against several antigens expressed in the prostate, indicating that PCa is a suitable target for immunotherapy. In this review, we will discuss PCa antigens that exhibit immunogenic features and/or have been targeted in immunotherapeutic settings with promising results, and we highlight the hurdles and opportunities for cancer immunotherapy.

The authors above then consider a collection of putative prostate antigens useful for applications of multiple approaches.

Antigen	*Function*	*Action*
PSA	PSA Serine protease which cleaves high molecular weight proteins into smaller peptides, resulting in the necessary liquification for spermatozoa to swim freely	Stimulates CTL Produces cytokines
PAP	PAP Protein tyrosine phosphatase which enhances the mobility of sperm	Stimulates CTL
PSMA	Folate hydrolase activity	Presented on cell surface. Elevated in PCa and HGPIN
PSCA	Unknown, overexpressed by most PCas	T-cell activation and proliferation
MUC-1	Limiting the activation of inflammatory response.	T-cell proliferation
NY-ESO-1	Unknown, expressed in a variety of tumors	CTLs and antibody-mediated responses
MAGE-A	Down-regulates p53 function through histone deacetylase recruitment	Stimulates CTLs in vivo
AKAP-4	Binding protein involved in cytoskeletal regulation and organization by affecting cyclic AMP-dependent protein kinase-A	Stimulated CTLs in vitro

Now for the dendritic cell targets they employ PAP as above as well as GM-CSF. The dendritic cells mature in a solution with a fusion protein (PA2024). The result is returned to the patient.

As Drake noted in 2009:

One of the few immunotherapy agents in late-stage development for prostate cancer is Sipuleucel-T (Dendreon Inc, Seattle WA, also Proverge). In this approach, patients undergo

plasmapheresis, and a personalized immunotherapy product is produced by culturing a patient's peripheral blood monocytes with a proprietary protein that couples granulocyte macrophage colony-stimulating factor with a target antigen (PAP). Phase I6,7 and phase III8 trials of Sipuleucel-T have been reported, with encouraging results. Clinical development of this agent is pivotal on a large (500 patients) randomized placebo-controlled phase III trial (ImPACT; Immunotherapy Prostate Adenocarcinoma Treatment) which completed accrual in October 2007, and for which additional survival data are expected sometime this year (see Note Added in Proof). In addition, considerable clinical development has focused on a viral vector approach in which PSA itself is targeted using sequential injections with recombinant vaccinia and fowlpox constructs.9 Here, both constructs have been engineered to include a number of costimulatory molecules in an effort to augment an immune response.

As Jahnisch et al note:

Dendritic cells (DCs) are professional antigen-presenting cells (APCs), which display a unique capacity to induce, sustain, and regulate T-cell responses. In tumor setting, DCs circulate through the blood and migrate to tumor tissues, where they interact with malignant cells. Immature DCs are particularly efficient in the uptake of tumorderived material. DC maturation is induced by tumorderived molecules such as heat shock proteins and high mobility- group box 1 protein as well as proinflammatory cytokines produced by various tumor-infiltrating immune cells.

During maturation DCs migrate from tumor tissues to T-cell-rich areas of secondary lymphoid organs, where they activate tumor-reactive CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ T cells. CD8+ CTLs efficiently recognize and destroy tumor cells, which expose peptides derived from tumor-associated antigens (TAAs) in the complex with human leukocyte antigen (HLA) class I molecules.

Clinical studies focusing on the adoptive transfer of cytotoxic effector cells revealed tumor regression in cancer patients [13]. CD4+ T cells recognizing peptides in the context of HLA class II molecules also play an important role in antitumor immunity. CD4+ T cells improve the capacity of DCs to induce CTLs by the interaction between CD40 on DCs and CD40 ligand on activated CD4+ T cells.

In addition, CD4+ T cells provide help for the maintenance and expansion of CTLs by secreting cytokines such as interleukin (IL)-2 and can eradicate tumor cells directly. Besides their extraordinary capacity to induce and stimulate T-cell responses, DCs efficiently improve the immunomodulatory and cytotoxic potential of natural killer cells, which essentially contribute to the elimination of tumor cells.

Furthermore, DCs can also directly mediate tumor-directed cytotoxicity. Owing to their various antitumor effects, DCs evolved as promising candidates for vaccination protocols in cancer therapy

Now as Mellman et al note:

While Provenge is clearly a cell-based therapy, there may be other mechanisms involved. Although the majority (66%) of survivors showed an antibody response to the fusion protein, the fraction of patients producing antibodies that recognized endogenous PAP was much lower (28.5%).

Moreover, T-cell responses to either the fusion protein or PAP were not associated with survival. These discrepancies might reflect a limitation of monitoring antitumor immune responses in the peripheral blood compared with the tumour microenvironment. However, they also raise the possibility that other undefined factors in the cellular product may have an important role. Further studies are required to understand the therapeutic mechanism of Provenge, and to define the impact of the different cell-processing procedures on the placebo product. The lack of tumour shrinkage, the criterion typically used to gauge the efficacy of cancer treatments, in the face of a survival benefit is surprising, but perhaps not unexpected for immunotherapy. As seen pre-clinically, an effect on pre-existing tumour due to immune manipulations can be delayed while an immune response develops.

Furthermore, biopsies of metastases after vaccination in some clinical trials revealed the presence of immune infiltrates that mediate tumour destruction in association with extensive edema, which may be followed by fibrosis46.

These histopathological findings suggest that monitoring tumour size alone may be inadequate for assessing the overall therapeutic effects of vaccination. As discussed later, these considerations apply to the evaluation of CTLA-4 antibody blockade, highlighting the need to modify tumour response criteria in light of new insights into the biology of immunotherapy.

Now Mellman et al make several key points as to the dendritic approach. First, RESIST approaches measure tumor size and in classic chemotherapy cases it does shrink. Yet as has been seen again and again in the more sophisticated and targeted approaches the shrinking takes time as the tumor, albeit present, it being attacked and killed off, albeit still visible on say a CAT Scan. Second, there is the putative supposition that there are other factors afoot. The latter we shall explore with the checkpoint examination.

13.3 CHECKPOINT TARGETS

Checkpoints are simply receptor-ligand pairs which when activated can inhibit the actions of T cells and other immune pathway actions. As Topalian et al note:

The rapid-fire clinical successes from blocking CTLA-4 and PD-1, the first checkpoint receptors to be discovered, have opened prospects for extending the potential of cancer immunotherapy by inhibiting more recently discovered checkpoint ligands and receptors. It is clear that, despite some commonalities, CTLA-4 and PD-1 have distinct patterns of expression, signaling pathways, and mechanisms of action. Although discovered over 20 years ago, there are still many unanswered questions about their biology, particularly in the context of cancer.

The authors continue:

The immune system recognizes and is poised to eliminate cancer but is held in check by inhibitory receptors and ligands. These immune checkpoint pathways, which normally maintain self-tolerance and limit collateral tissue damage during anti-microbial immune responses, can be co-opted by cancer to evade immune destruction. Drugs interrupting immune checkpoints, such as anti-CTLA-4, anti-PD-1, anti-PD-L1, and others in early development, can unleash anti-tumor immunity and mediate durable cancer regressions. The complex biology of immune checkpoint pathways still contains many mysteries, and the full activity spectrum of checkpoint-blocking drugs, used alone or in combination, is currently the subject of intense study.

Thus, the issue would be; what other check points are there and how can they be addressed? From Kono we have the following Table which presents some putative targets:

Target	Biological function	Antibody (fusion protein)	Phase	Cancer type
CTLA4	Inhibitory receptor	Ipilimumab	FDA approved Phase II and III	melanoma, multiple cancers
PD1	Inhibitory receptor	MDX-1106 MK3475 CT-011 AMP-224	Phase I/II Phase I Phase I Phase I	melanoma, renal, lung multiple cancers multiple cancers multiple cancers
PDL1	Ligand for PD1	MDX-1105	Phase I	multiple cancers
LAG3	Inhibitory receptor	IMP321	Phase II	breast cancer
B7-H3	Inhibitory ligand	MGA271	Phase I	multiple cancers
B7-H4	Inhibitory ligand			Preclinical
TIM3	Inhibitory receptor			Preclinical

From the recent work of Beer et al:

Ipilimumab is a fully human monoclonal immunoglobulin G1 antibody that increases antitumor T-cell responses by binding to cytotoxic T-lymphocyte antigen 4.17-19 Blocking by ipilimumab of the T-cell negative regulator cytotoxic T-lymphocyte antigen 4 allows CD28 and B7 interactions, which result in T-cell activation; proliferation; tumor infiltration; and ultimately, cancer cell death. Treatment with ipilimumab, as a single agent or in combination with dacarbazine, provided significant survival benefit in two phase III trials of advanced melanoma. Of note, approximately 20% of ipilimumab-treated patients with melanoma experienced longterm survival

We have seen this in detail when examining the melanoma therapeutic approaches. Now the application of this to PCa is interesting and challenging. Melanoma is an aggressive and rapidly growing cancer and it is well known that it often evokes an immune response when examined on biopsy. In contrast PCa is quite different. Melanoma is derived from melanocytes which have developed from the neural crest. PCa is exocrine glandular. The results of the Beer trial were not conclusive.

More recently Schweizer and Drake (2014) noted:

Since the approval of sipuleucel-T for men with metastatic castrate resistant prostate cancer in 2010, great strides in the development of anti-cancer immunotherapies have been made. Current drug development in this area has focused primarily on antigen specific [i.e. cancer vaccines and antibody based therapies)] or checkpoint inhibitor therapies, with the checkpoint inhibitors perhaps gaining the most attention as of late.

Indeed, drugs blocking the inhibitory signal generated by the engagement of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) found on T-cells has emerged as potent means to combat the immunosuppressive milieu. The anti-CTLA-4 monoclonal antibody ipilimumab has already been approved in advanced melanoma and two phase III trials evaluating ipilimumab in men with metastatic castrate-resistant prostate cancer are underway.

A phase III trial evaluating ProstVac- VF, a poxvirus-based therapeutic prostate cancer vaccine, is also underway. While there has been reason for encouragement over the past few years, many questions regarding the use of immunotherapies remain.

Namely it is unclear what stage of disease is most likely to benefit from these approaches, how best to incorporate said treatments with each other and into our current treatment regimens and which therapy is most appropriate for which disease. Herein we review some of the recent advances in immunotherapy as related to the treatment of prostate cancer and outline some of the challenges that lie ahead.

More recently Martin et al noted:

Primary prostate cancers are infiltrated with PD-1 expressing CD8+ T cells. However, in early clinical trials, men with mCRPC did not respond to PD-1 blockade as a monotherapy. One explanation for this unresponsiveness could be that prostate tumors generally do not express PD-L1, the primary ligand for PD-1.

However, lack of PD-L1 expression in prostate cancer would be surprising, given that PTEN loss is relatively common in prostate cancer and several studies have shown that PTEN loss correlates with PD-L1 up-regulation - constituting a mechanism of innate immune resistance. This study tested whether prostate cancer cells were capable of expressing PD-L1, and whether the rare PD-L1 expression that occurs in human specimen's correlates with PTEN loss…These studies show that some prostate cancer cell lines are capable of expressing PD-L1.

However, in human prostate cancer, PTEN loss is not associated with PD-L1 expression, arguing against innate immune resistance as a mechanism that mitigates anti-tumor immune responses in this disease.

Unfortunately, the results are less than positive. They seem to agree with the prior results.

13.4 OPTIONS

There are many options available for dealing with PCa but the efficacy of these known options is at best problematic. Yoo et al have summarized the recent (2016) immunotherapeutic options for PCa. They note:

Despite advances in treatment of prostate cancer, curative therapy is not yet available for CRPC. Novel therapeutic options have thus been sought, and vaccines, immunotherapy, and gene based therapy are considered to be attractive candidates in this respect.

Up to now, sipuleucel-T is the only such treatment approved by the Food and Drug Administration.

In this section, the authors will briefly introduce investigational vaccines, immunotherapy, and gene-based therapy for CRPC.

5.1. Vaccine

GX301is a dual-adjuvant telomerase vaccine. GX301 is reported to be safe and highly immunogenic in patients with prostate cancer. A Phase II randomized trial is underway.

Prostvac is a vector based therapeutic cancer vaccine. A Phase II study reported that prostvac was well tolerated and it improved overall survival compared with control vectors (25.1 months vs. 16.6 months) in patients with minimally symptomatic CRPC. However, another Phase II study, which evaluated the effect of the combination of docetaxel and prostvac, failed to show improvements in overall survival; this lack of positive results may be due to limited accrual of patients. Investigation on the relative efficacy of simultaneous versus sequential docetaxel þ prostvac is currently ongoing.

DCVAC is an autologous dendritic cell-based vaccine. In a Phase I and II trial, combination chemoimmunotherapy with DCVAC and docetaxel resulted in longer than expected survival (19 months vs. 11.8 months) without significant complications. A Phase III study, evaluating the merits of DCVAC when added to standard chemotherapy, is due to commence.

13.5 IMMUNOTHERAPY

Ipilimumab[64] *is a monoclonal antibody that blocks the activity of CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and was approved by the Food and Drug Administration for the treatment of melanoma in 2011. As preclinical and clinical studies suggested that radiotherapy might activate the immune system in patients with prostate cancer, a Phase III trial of ipilimumab in addition to radiotherapy for metastatic CRPC patients was initiated. However, this Phase III study did not show any improvement in overall survival after radiotherapy followed by ipilimumab, compared with radiotherapy followed by placebo.*

[64] Also, known as Yervoy.

Currently, combination trials with abiraterone, ADT, sipuleucel-T, and prostvac are underway. 177Lu-J591, a humanized monoclonal antibody, was primarily developed in a radiolabeled form for PET, binding to the extracellular domain of prostate-specific membrane antigen (PSMA). After binding to PSMA, the 177Lu-J591ePSMA complex undergoes endocytosis and is accumulated in prostate cancer cells.

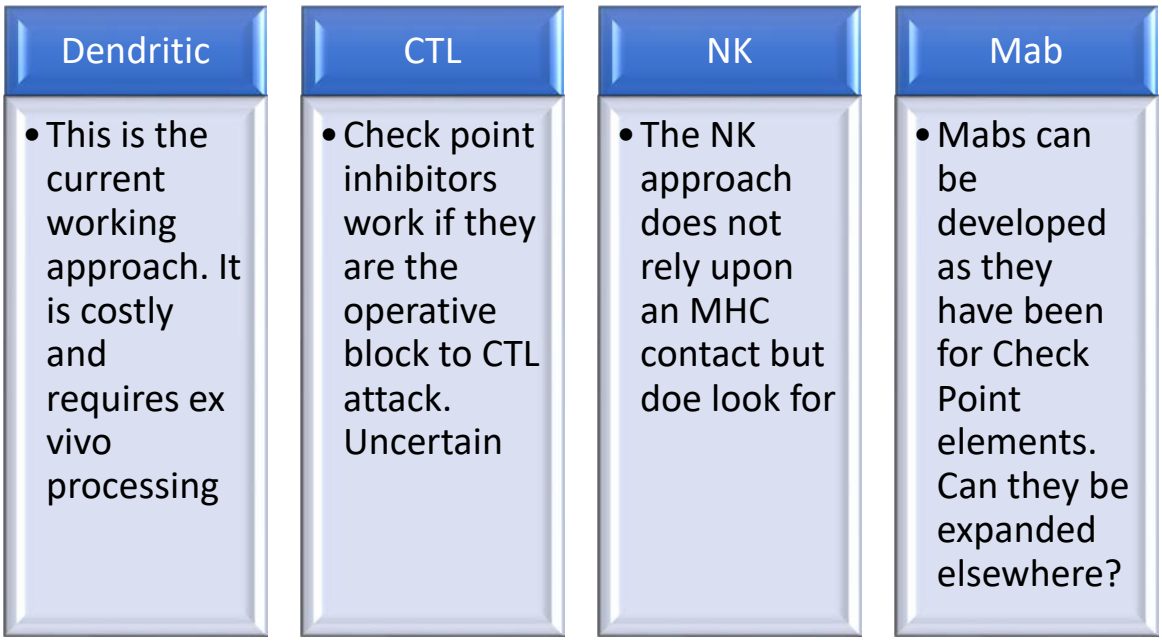
In this regard, 177Lu-J591J591 is considered to be a potential carrier for cytotoxic drug conjugates to maximize therapeutic effectiveness43 and a promising agent for radioimmunotherapy. Currently, a Phase 2 clinical trial is in the process of patient recruitment.

13.6 GENE-BASED THERAPY

Olaparib, recently approved for treating ovarian cancer with BRCA1/2 mutations, is a poly-ADT-ribose polymerase inhibitor. Poly-ADT-ribose polymerase is involved in the DNA repair process, and genomic aberrations observed in CRPC are thought to confer sensitivity to poly-ADTribose polymerase inhibitors. In recent studies, olaparib showed a considerable response rate of 33% in post-docetaxel prostate cancer patients with defects in DNA repair genes,44 and a Phase II trial has commenced.

Thus, the only accepted approach is the dendritic approach that have been approved. The checkpoint approach has not yield any significant positive result although trial continue.

Let us review the options.



CRISPR	CAR T	Gene Drive	CIK
• We know that CRISPRS can insert and/or alter genes and we know some cells can be altered to express Ag which can be addressed.	• The CAR T cell approach works well for some hematological cancers. CD 19 is a typical market. The question is; is there a similar PCa marker or can we insert such a marker?	• The Gene Drive approach would attempt to insert a new genetic structure in CTLs and force them forward.	• CIK has been found useful in some hemetalogical cancers and we ask if this may work here as well.

14 OTHER INFLUENCES

We now examine two other influences in dealing with the immune system; the microbiome and inflammation. Both have become more integral in terms of treatment and efficacy. Some recent papers as summarized in Nature have discussed the progress in understanding this phenomenon. Now the Nature piece states:

Preclinical mouse models have shown that the gut microbiome can modulate therapeutic responses to cancer therapies. Yet, this has not been extensively characterized in humans. Two studies now propose that the gut microbiome is an important host factor that determines the response and primary resistance to anti-programmed cell death protein 1 (PD1) immunotherapy in patients with cancer. "the clinical response to PD1 blockade could be predicted by the composition of the gut microbiome" Both groups initially sought to determine whether the clinical response to PD1 blockade could be predicted by the composition of the gut microbiome. To achieve this, faecal samples were collected from patients with either melanoma, non-small-cell lung cancer (NSCLC) or renal cell carcinoma (RCC) before and after commencement of immunotherapy. Metagenomic shotgun sequencing was then used to quantify bacterial species. A common finding was that high diversity of the gut microbiome correlated with prolonged progression-free survival (PFS) following PD1 inhibition.

The papers are by Routy et al and the other by Gopalakrishnan et al. Routy et al note:

Immune checkpoint inhibitors (ICI) targeting the PD-1/PD-L1 axis induce sustained clinical responses in a sizeable minority of cancer patients. Here, we show that primary resistance to ICI can be due to abnormal gut microbiome composition. Antibiotics (ATB) inhibited the clinical benefit of ICI in patients with advanced cancer. Fecal microbiota transplantation (FMT) from cancer patients who responded to ICI (but not from non-responding patients) into germ-free or ATB-treated mice ameliorated the antitumor effects of PD-1 blockade. Metagenomics of patient stools at diagnosis revealed correlations between clinical responses to ICI and the relative abundance of Akkermansia muciniphila. Oral supplementation with A. muciniphila post-FMT with non-responder feces restored the efficacy of PD-1 blockade in an IL-12-dependent manner, by increasing the recruitment of $CCR9^{+}CXCR3^{+}CD4^{+}$ T lymphocytes into tumor beds.

There clearly are a multiplicity of dimensions in using the immune system to combat cancers. It is essential to view this as a systems problem, understanding that we are a bit ignorant of all of its dimensions.

14.1 MICROBIOME

It can be said that every mammal is a concatenation of a multiplicity of species generally living in harmony. A human exists side by side with commensurate bacteria, viruses, fungi, that help the human maintain normal homeostasis. They assist in balancing pH, in breaking down carbohydrates, and in interacting with the immune system. At the extreme there are exogeneous microorganisms which can be harmful to the prime organism and the resident microorganisms can become a part in some sense of the immune system of that organism. This would even

include the collection of such cohabitating micro-organisms even controlling aberrant cell growth as seen in cancers.

The NIH has an extensive Microbiome Project[65]. As NIH states:

Microscopic study of the healthy human body has demonstrated that microbial cells outnumber human cells by about ten to one. Until recently though, this abundant community of human-associated microbes remained largely unstudied, leaving their influence upon human development, physiology, immunity, and nutrition almost entirely unknown. The NIH Common Fund Human Microbiome Project (HMP) was established with the mission of generating research resources enabling comprehensive characterization of the human microbiota and analysis of their role in human health and disease. The information generated by HMP is made available worldwide for use by investigators and others in efforts to understand and improve human health.

It appears that the NIH project has significant current limits. We shall explore dimensions outside of the NIH study, namely the interaction of the microbiome with cancer.

The microbiome is a terms to describe the collection of microbiological entities, bacteria, fungi, viruses, that inhabit the normal health individual and often play a key role in homeostasis. Soon after the discovery of bacteria, it was thought that any foreign microorganism may have deleterious effects. It soon changed since the colon is filled with microorganisms that assist in the digestion and utilization of foods. Recently the doctrine that urine was sterile and that anything the indicated a microorganism was present was a defect was overthrown (see Ainsworth).

Moreover a growth of studies showing that the microbiome is efficacious in fighting cancers has been developed. At one extreme is the importance of the microbiome in managing types of chemotherapy to the critical nature of the microbiome in facilitating the immune system early on in fighting cancer cells, and especially cancer stem cells. The microbiome may very well present an added tool to facilitating the body's own systems in fighting a variety of malignancies.

In this note we examine some of the recent research and

As Vogtmann and Goedert have recently noted:

Human microbiome research has garnered substantial attention, both by scientists and the media. The human microbiome refers to the collective genome of all bacteria, archaea, fungi, protists, and viruses residing in and on the human body. Made feasible by high throughput, next-generation deep sequencing of DNA, as well as expanding computational and bioinformatics support, the microbiome is a conceptual quantum leap from detection and identification of individual microbes to characterization of entire microbial communities, including both pathogenic and commensal microbes that have not yet been cultured or otherwise detected. Differences among individuals in our co-dependent relationship with the microbiota is

[65] https://commonfund.nih.gov/hmp

postulated to modulate susceptibility to many malignancies via several pathways, including nutrition, detoxification, metabolism, hormonal homeostasis, immune tolerance, and especially inflammation

The above makes a significant point. Namely, the availability of new and improved measurement devices and methods allow us to look at the microbiome in significant detail.

As Eureka notes:

Enterococcus faecalis 2001 is a probiotic lactic acid bacterium and has been used as a biological response modifier (BRM). From physiological limitation of bacterial preservation in storage and safety, the live E. faecalis 2001 has been heat-treated and the BRM components containing high level of β-glucan, named EF-2001, were prepared. Method: The heat-treated EF-2001 has been examined for the antioxidative potential for radical scavenging and anti-tumor activities as well as immune-enhancing response in mice.

Lymphocyte versus polymorphonuclear leukocyte ratio was increased in mice upon treatment with EF-2001. The number of lymphocytes was increased in the EF-2001-treated group. In the mice bearing two different Ehrlich solid and Sarcoma-180 carcinomas, the treatment with EF-2001 resulted in anti-tumor action. Tumor-suppressive capacity upon treatment with EF-2001 was significantly increased compared to normal controls. Results: During the time interval administration of 5 weeks between the priming and secondary administration of EF-2001, the expression and production levels of TNF-α were also observed in the EF-2001administered mice. Additionally, anti-tumor activity examined with the intravenous administration of EF 2001 with a 34 time intervals was also observed, as the growth of Sarcoma180 cells was clearly inhibited by the EF-2001. Conclusion: From the results, it was suggested that the immune response is enhanced due to antioxidative activity caused by the EF-2001 and anti-tumor activity by NK cells and TNF-α.

The microbiome is the concatenation of organisms in the multiplicity of organism in the human body. The interaction of these microorganisms and the human cells, local and distant from their presence, presents an overwhelming complex system to be considered.

As we have noted, the microbiome is that collection of micro-organisms which can co-exist with the human organism and in the process not generate an immune response including inflammation as well as contribute towards a benign homeostasis. We have recently seen an increased interest in the microbiome as an adjunct in cancer therapy as well as a putative cause of many cancers.

As Cho and Blazer note:

Interest in the role of the microbiome in human health has burgeoned over the past decade with the advent of new technologies for interrogating complex microbial communities. The large-scale dynamics of the microbiome can be described by many of the tools and observations used in the study of population ecology. Deciphering the metagenome and its aggregate genetic information can also be used to understand the functional properties of the microbial

community. Both the microbiome and metagenome probably have important functions in health and disease; their exploration is a frontier in human genetics.

Part of the issue is that the microbiome is generally neglected when examining cancers. Yet it has been found that it can in some cases facilitate the treatments and in others inhibit it. As Lloyd-Price et al note:

Microbiomes regularly show a large degree of interpersonal diversity even in the absence of disease. This complicates the identification of simple microbial constituents or imbalances that either cause disease or reflect a diseased state. An understanding of the properties of a healthy microbiome, and the many different microbial ecologies that are encountered in the absence of overt disease, is therefore a necessary first step to identifying and correcting microbial configurations that are implicated in disease. In this review, we use "healthy" to refer to the absence of any overt disease.

It is this diversity between people that makes it a difficult system to assess. In addition to person to person diversity is the temporal diversity in a single person. Furthermore is the diversity across organisms in the body not to mention the complexity of interactions between microorganisms and their responses. The authors continue;

Most available data describe the gut microbiome and so many of the findings discussed here are from this area, though most principles apply to microbial habitats throughout the body. Early research into the ecology of the microbiome sought to identify a "core" set of microbial taxa universally present in healthy individuals who lack overt disease phenotypes, under the hypothesis that the absence of such microbes would indicate dysbiosis; but studies of ecological diversity among healthy individuals revealed sufficient variation in the taxonomic composition of the microbiome to rapidly render such a hypothesis unlikely.

The microbiome is present in all organs; the gastro system, the oral cavity, the bladder and even the hematological system. As Thaiss et al state:

The intestinal microbiome is a signalling hub that integrates environmental inputs, such as diet, with genetic and immune signals to affect the host's metabolism, immunity and response to infection.

The haematopoietic and non-haematopoietic cells of the innate immune system are located strategically at the host–microbiome interface. These cells have the ability to sense microorganisms or their metabolic products and to translate the signals into host physiological responses and the regulation of microbial ecology. Aberrations in the communication between the innate immune system and the gut microbiota might contribute to complex diseases.

The innate immune system, with the collection of Pattern Recognition Receptors, such as the Toll Like Receptors, TLR, are integral in early recognition of and response to such microorganisms as those found in a microbiome. Then one may wonder why there is not a continual battle between the immune system and the microbiome. Why one may wonder is the

mouth not in a continual inflammatory state with the ongoing release of chemokines and cytokines, why the intestinal system does not have a similar ongoing battle. Thaiss et al continue:

The past two decades witnessed a revolution in our understanding of host–microbial interactions that led to the concept of the mammalian holobiont — the result of co-evolution of the eukaryotic and prokaryotic parts of an organism. The revolution required two paradigm shifts that had a tremendous impact on their respective fields.

The first occurred during the late 1990s with the discovery of pattern recognition receptors (PRRs) in the innate immune system that sense microorganisms through conserved molecular structures. Several families of PRRs and their signalling pathways are now known, including the Toll-like receptors (TLRs), the nucleotide-binding oligomerization (NOD)-like receptors (NLRs), the RIG-I-like receptors, the C-type lectin receptors, the absent in melanoma 2 (AIM2)-like receptors and the OAS-like receptors1. These sensors are expressed by a variety of cellular compartments and constitute a continuous surveillance system for the presence of microorganisms in tissues.

The second shift occurred fewer than 10 years later and was driven by the culture-independent characterization of the microbiome2 — the entirety of the microorganisms that colonize the human body and their genomes. Because of the enormous number of microorganisms that reside on the surface of the body — the skin and the gastrointestinal, respiratory and urogenital tracts — it seemed improbable that innate immune recognition of microorganisms could be coupled to the immediate initiation of immune responses against them without leading to overt, organism-wide inflammation and its damaging effects. It was therefore hypothesized that microbial sensing at the body surface needs to be tightly controlled to ensure a symbiotic relationship between the host and its indigenous commensal microorganisms3, while allowing for the initiation of a rapid, sterilizing immune response on penetration of microorganisms into non-colonized sites. This idea was developed further after the realization that host–microbiota mutualism is lost in the absence of innate immune recognition of commensal microorganisms, with detrimental consequences for health. The crosstalk between innate immunity and the microbiome is now known to extend far beyond the achievement of a careful balance between tolerance to commensal microorganisms and immunity to pathogens.

The innate immune system has often been looked upon as a poor cousin to the adaptive system Yet its relationship to and with the microbiome presents a complex system problem that may offer new dimensions to how to manage a stable biome while addressing the changes one sees in a malignant transformation. Thaiss et al conclude:

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Thus the stability between the microbiome and the innate system will be a key factor in comprehending its efficacy.

14.1.1 Interaction with Immune System

The microbiome, the benign and pathologic, are inherently all presenters of antigens to the immune system. In the case of the benign and common microbiome, some form of stasis is reached by various means such as physical isolation of antigens. The human body has a multiplicity of cells with pattern recognition receptors which are on a constant look-out for antigens to then be activated and provide an effective immune response. That would be counterproductive for that part of the microbiome which is part of the homeostatic system.

14.1.2 Example of Innate System with Microbiome

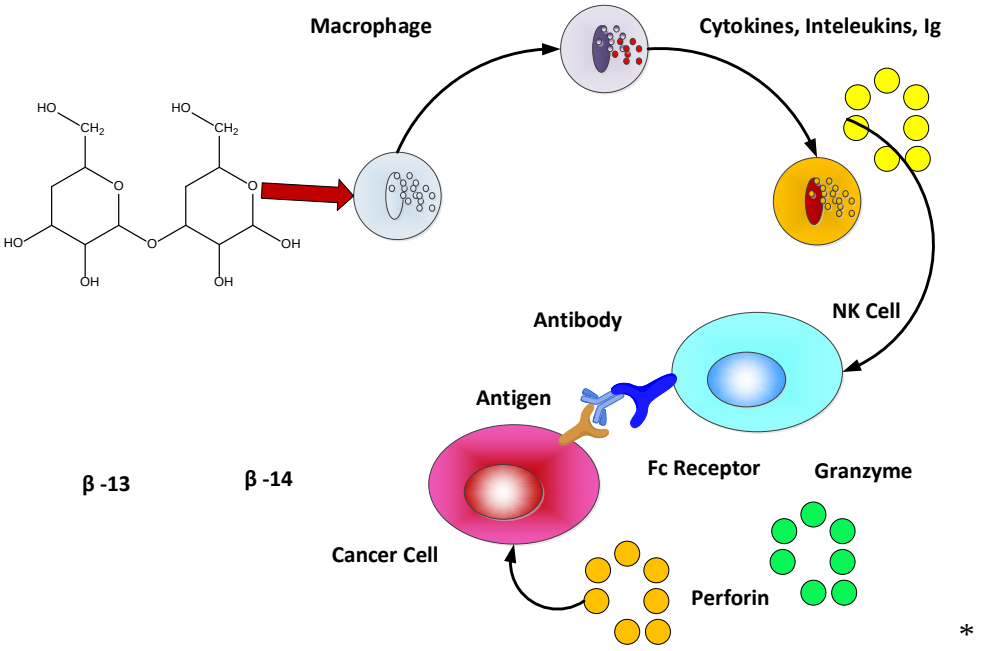
The interest in this topic as noted earlier was driven by some recent work in the intersection of the microbiome and the innate system. As Gu et al note:

Enterococcus faecalis 2001 is a probiotic lactic acid bacterium and has been used as a biological response modifier (BRM). From physiological limitation of bacterial preservation in storage and safety, the live E. faecalis 2001 has been heat-treated and the BRM components containing high level of β-glucan, named EF-2001, were prepared…The heat-treated EF-2001 has been examined for the antioxidative potential for radical scavenging and anti-tumor activities as well as immune-enhancing response in mice. Lymphocyte versus polymorphonuclear leukocyte ratio was increased in mice upon treatment with EF-2001. The number of lymphocytes was increased in the EF-2001-treated group.

In the mice bearing two different Ehrlich solid and Sarcoma-180 carcinomas, the treatment with EF-2001 resulted in anti-tumor action. Tumor-suppressive capacity upon treatment with EF-2001 was significantly increased compared to normal controls…During the time interval administration of 5 weeks between the priming and secondary administration of EF-2001, the expression and production levels of TNF-α were also observed in the EF- 2001-administered mice. Additionally, anti-tumor activity examined with the intravenous administration of EF 2001 with a 34 times interval was also observed, as the growth of Sarcoma180 cells was clearly inhibited by the EF-2001…

From the results, it was suggested that the immune response is enhanced due to antioxidative activity caused by the EF-2001 and anti-tumor activity by NK cells and TNF-α.

We can depict this effect as shown below. Here we have a definable mechanism wherein the activation of a product in the microbiome can then identify and attack an early stage malignant cell.



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The above raises several interesting questions. First, we need the details of the interaction. Second, understanding the details can we manage to force this interaction, make it more aggressive, and facilitate it use in a cancer modulation process.

Zitvogel et al note:

The human gut microbiome modulates many host processes, including metabolism, inflammation, and immune and cellular responses. It is becoming increasingly apparent that the microbiome can also influence the development of cancer. In preclinical models, the host response to cancer treatment has been improved by modulating the gut microbiome; this is known to have an altered composition in many diseases, including cancer.

In addition, cancer treatment with microbial agents or their products has the potential to shrink tumours. However, the microbiome could also negatively influence cancer prognosis through the production of potentially oncogenic toxins and metabolites by bacteria. Thus, future antineoplastic treatments could combine the modulation of the microbiome and its products with immunotherapeutics and more conventional approaches that directly target malignant cells.

In fact, the microbiome not only modulates but it can initiate and facilitate the overall process of innate immune response.

14.1.3 Innate Lymphoid Cells

We first consider the collection of cells called the Innate Lymphoid Cells, ILC. These cells, not fully understood, appear to play a critical role in many microbiome related processes. However their function and even their identity is still under study and for many their very existence is unknown.

From Abbas et al:

Innate lymphoid cells (ILCs)… are bone marrow–derived cells with lymphocyte morphology that were discovered as cells that produced cytokines similar to those made by T cells but lacked TCRs. We call them "lymphoid cells," not "lymphocytes," because they do not express clonally distributed diverse antigen receptors like the T lymphocytes they otherwise resemble.

There are different subsets of ILCs that arise from the same common lymphoid precursor that gives rise to B and T cells, but the precise steps in ILC development are not fully understood, especially in humans. It is clear that during their development, there are branch points giving rise to three different "helper" subsets of ILCs, which function mainly by secreting different types of cytokines, similar to CD4+ helper T cell subsets, and a separate branch giving rise to natural killer (NK) cells, which function as cytotoxic effectors in addition to secreting the cytokine interferon-γ, similar to CD8+ cytotoxic T lymphocytes…

Three subsets of innate lymphoid cells, called ILC1, ILC2, and ILC3, produce different cytokines and express different transcription factors, analogous to the Th1, Th2, and Th17 subsets of CD4+ T lymphocytes. The cytokines each subset produces determine the roles of these cells in defense, and the transcription factors are required for differentiation and function of each of the three subsets. ILC1s produce IFN-γ and express the transcription factor T-bet, like Th1 cells. ILC2s produce IL-5, IL-9, and IL-13, and express the transcription factor GATA-3, like Th2 cells. ILC3s produce IL-22 and/or IL-17 and express the transcription factor RORγt, like Th17

cells. Because ILCs do not express T cell receptors, they must be activated by different mechanisms than helper T cells to produce these cytokines. The best defined stimuli for ILC cytokine production are other cytokines, released in the context of innate responses to infections and tissue damage; each ILC subset is activated by different cytokines.

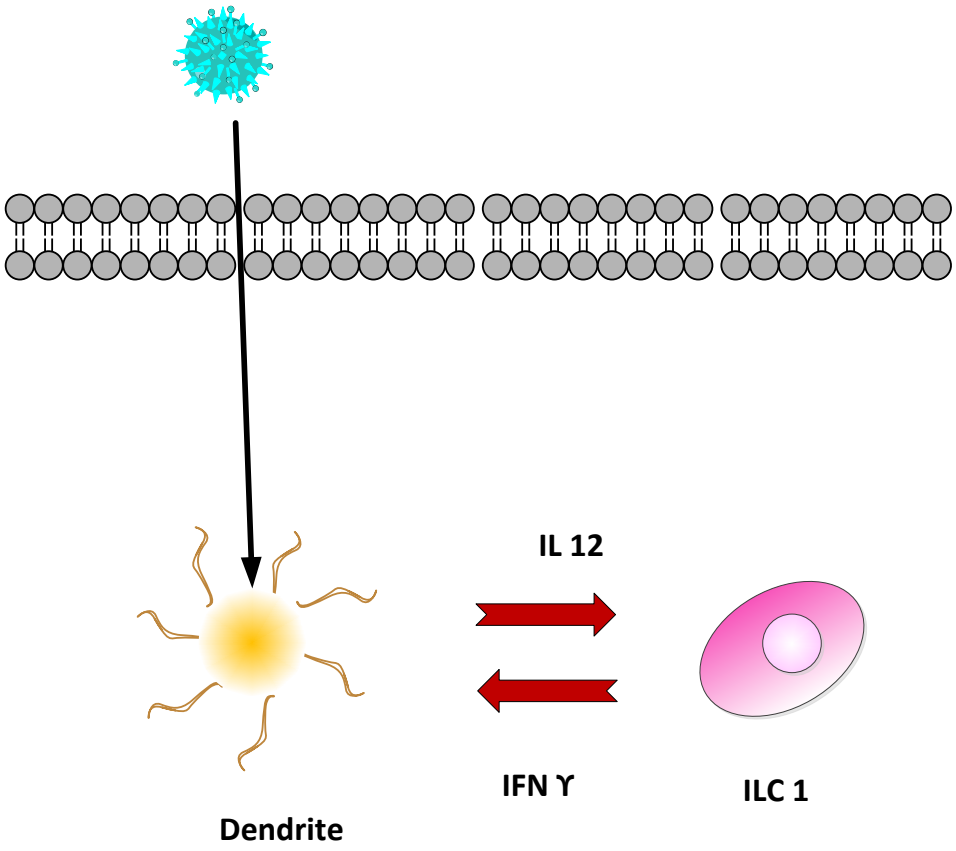
They continue:

ILC subsets may participate in host defense against distinct pathogens and also may be involved in inflammatory disorders. ILC1s are likely important for defense against intracellular microbes. ILC2s are important for defense against helminthic parasites, and they also may contribute to allergic diseases. ILC3s are found at mucosal sites and participate in defense against extracellular fungi and bacteria, as well as in maintaining the integrity of epithelial barriers. Lymphoid tissue–inducer (LTi) cells are a subtype of ILC3s, which, in addition to secreting IL-17 and IL-22, also express the membrane molecule lymphotoxin-α and secrete TNF, both of which are required for the normal development of lymphoid organs.

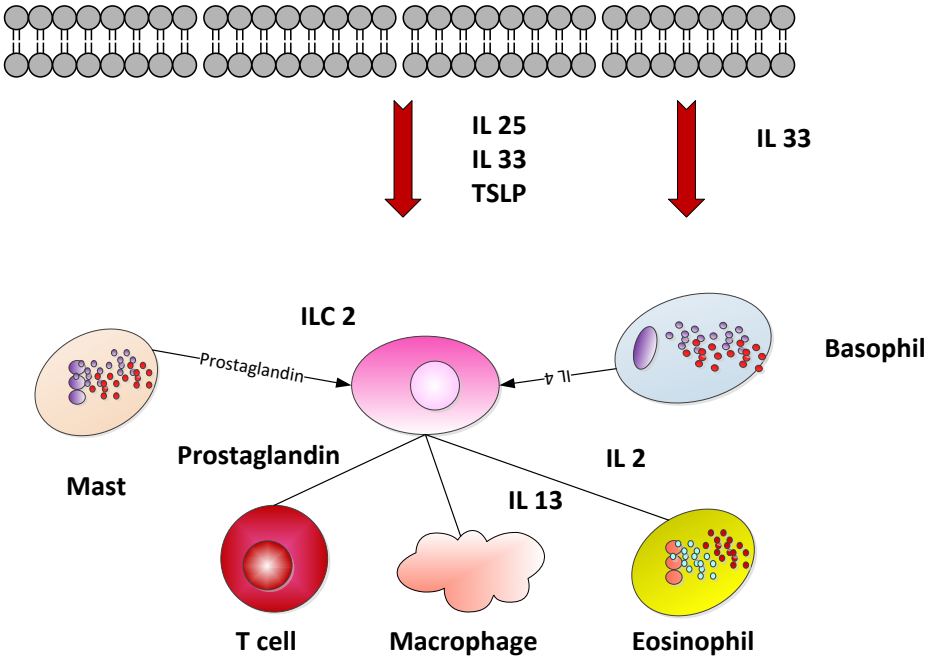
The contribution of ILCs to host defense has been difficult to establish because it has not been possible to selectively eliminate these cells or their cytokines without impacting the analogous T lymphocytes as well. The feature of ILCs that makes them potentially important for early host defense is that they are always resident in epithelial barrier tissues, poised to react against microbes that breach those barriers. In contrast, T cells circulate through secondary lymphoid organs and migrate into tissues only after they are activated and differentiate into effector cells, a process that may take several days after encounter with a microbe. It is, therefore, possible that ILCs are early responders to microbes that colonize tissues, and over time this role is assumed by differentiated effector T cells, which are which are more specific and produce larger amounts of cytokines.

The three ILCs interact as follows:

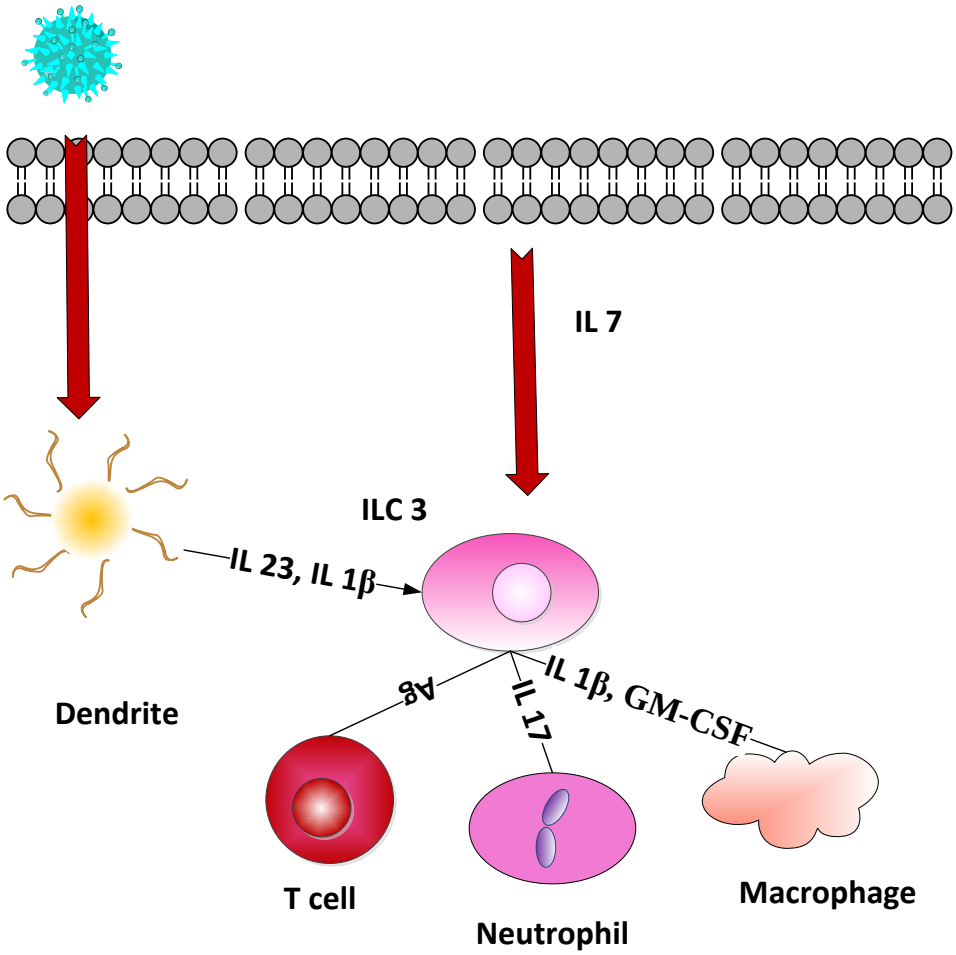
ILC 1 is activated by some organism through the cell wall via a dendrite. IL 12 activates the IL 1 and in turn the IL 1 produces a feedback IFN γ.



ILC 2 is depicted below. Here we have a basophil activation along with a complementary mast cell. ILC 2 then in turn turns up the T cells, Macrophages and eosinophils.



Finally we have the ILC 3 cells activated by a flagellin via a dendritic cell, and co-stimulated by IL 7 and then it activates the T cell, the macrophage and now a neutrophil.



We find that the ILCs are apparently facilitators of the microbiome and its interaction with the innate immune system. It is not at all clear what the temporal characteristics are but generally the innate system does have a near real time response mechanism. The ILCs may be activated via an activated microbiome. As we will also note later that Guglielmi discusses the complex interaction between the microbiome, phagosome, phage or viral elements, and in turn the immune system.

14.1.4 Cancer

It has become mora apparent that many infections and resulting inflammatory effects are a basis for the initiation and development of a multiplicity of cancers. Now infections are basically the introduction of and proliferation of exogenous pathogenic organisms in the human biome. They are organisms, bacterial, viral or fungal, that initiate some possible immune response. They may have a PRR, pattern recognition receptor such as a TLR, type response. Inflammation is the immune system response which may be the result of any infection or even a self-generate an immune response such as a self-immunity. Finally we separate obesity as a major cause of cancer separate and apart. Obesity establishes an environment which results in inflammatory like

responses. It places significant stress upon the organisms, in this case the human body, which, over time, result in changes leading towards a malignancy.

The effects that the above induce may be the result of genetic changes, deletions, additions or translocations, or the impact may be epigenetic via some methylation change or acetylation change, or it may even be a miRNA induction or suppression. Clarity of cause and effect has yet to be established.

As Garrett notes:

Microbiota contribute to carcinogenesis, whether by enhancing or diminishing a host's risk, fall into three broad categories: (i) altering the balance of host cell proliferation and death, (ii) guiding immune system function, and (iii) influencing metabolism of host-produced factors, ingested foodstuffs, and pharmaceuticals (Fig. 1). Assigning microbial communities, their members, and aggregate biomolecular activities into these categories will require a substantial research commitment. …

Bona fide oncomicrobes—microbes that trigger transformation events in host cells—are rare. Beyond the 10 IACR-designated microbes, there are a handful of other microorganisms with robust but fewer aggregate data supporting their role in human carcinogenesis. As many of these and their carcinogenic mechanisms have been recently reviewed, select activities representing common pathways by which microbes influence cancer will be highlighted. Human onco-viruses can drive carcinogenesis by integrating oncogenes into host genomes. Human papillomaviruses (HPV) express onco-proteins such as E6 and E7.

Data from recent genomic analyses of HPV+ cervical cancers suggest that viral integration also selectively triggers amplification of host genes in pathways with established roles in cancer. Microbes also drive transformation by affecting genomic stability, resistance to cell death, and proliferative signaling. Many bacteria have evolved mechanisms to damage DNA, so as to kill competitors and survive in the microbial world.

14.1.4.1 Infections

Infections and the impact of the microbiome has become a compelling area of study in cancer epidemiology. As Martel et al have concluded:

In view of the high mortality rate of infection associated cancers, the fraction of cancer deaths attributable to infections is probably higher than the 16·1% that our study generated. Although a full investigation of cancer death due to infection is beyond the scope of this report, we can estimate the mortality burden by applying the PAFs to the 7·5 million cancer deaths that occurred in 2008. These calculations suggest that 1·5 million cancer deaths were attributable to infectious agents, or roughly one in five deaths due to cancer worldwide

Simply they estimated that 20% of cancer deaths were related to some form of overt infection. This does not include microbiome distortions, those changes that result in DNA reconfiguration.

Their analysis is somewhat dated, not due to the analytical approach but to what impact the microbiome truly has on cancer development.

14.1.4.2 Inflammation

In contrast we also see that chronic inflammation, a process caused by some irritant not necessarily diagnosable as an overt infection has a dramatic effect. For example, as Thaiss et al note:

The idea that chronic inflammation drives carcinogenesis has been widely established in various tissues. For example, hepatocellular carcinomas arise in people with chronic hepatitis, colorectal cancer can occur in people with longstanding untreated IBD and Marjolin's ulcers develop on chronically inflamed skin. The presence of bacteria at tumour sites was first described more than a century ago, so it is surprising that the role of the microbiota in tumourigenesis has only recently been recognized. Colorectal carcinogenesis is triggered by a combination of microbiota- and host-dependent mechanisms. Certain bacteria promote carcinogenesis directly, through the secretion of substances that elicit DNA damage.

The colon is a likely place for various pathogens to flourish. In fact the microbiome of the colon is an ever changing environment and the presence of certain oncogenic products is well known as causal for malignancies. The classic Vogelstein model depicts how this may occur. The author continues:

Prominent examples include the excessive release of nitric oxide from immune cells that is triggered by Helicobacter hepaticus, the production of reactive oxygen species by Enterococcus faecalis and the secretion of an enterotoxin by Bacteroides fragilis, which activates the oncogene c-MYC. Other bacteria drive carcinogenesis indirectly by sustaining a proinflammatory microenvironment, such as the production by Fusobacterium nucleatum of the virulence factor FadA, which increases the paracellular permeability of colonic epithelial cells. Inflammation might also promote community-level alterations in the microbiome and facilitate bacterial translocation into neoplastic tissue, which further promotes the expression of inflammatory cytokines and leads to the increased growth of tumours. Dysbiosis that arises in the absence of NLRP6 promotes the development of cancer through IL-6-induced epithelial proliferation.

Reactive Oxygen Species, ROS, are well know instigators of DNA damage and in turn mutagenic effects leading to malignancies. However, these often occur at low levels in inflammatory conditions and here to we would see such damage. Continuing:

The influence of the microbiota on innate immunity has been shown to affect the host response to cancer therapy. For example, germ-free mice and mice that are treated with antibiotics both show a diminished response to immunotherapy by CpG oligonucleotides and chemotherapy owing to the impaired function of myeloid-derived cells in the tumour microenvironment. Furthermore, commensal Bifidobacterium enhances immunity to tumours through antibodies directed against programmed cell death 1 ligand 1 (PD-L1) through the augmentation of dendritic-cell function. These studies might open up a fascinating avenue of research to prevent cancer and develop cancer therapeutics through manipulation of the microbiota.

The PD-L1 effect is critical. Cancers have the ability to present PD-L1 and to block PD-1 from activation and inhibiting the immune system from destroying the cancer cell. There is a plethora of immunotherapy doing the same type of function done in the microbiome. The question is; why doe the microbiome achieve this for only a select types of malignant cells?

As Garrett notes:

Mechanisms by which microbes influence cancer development and progression.

(A) Bacterial toxins can directly damage host DNA. Bacteria also damage DNA indirectly via host produced reactive oxygen and nitrogen species. When DNA damage exceeds host cell repair capacity, cell death or cancer-enabling mutations occur.

(B) b-Catenin signaling alterations are a frequent target of cancer-associated microbes. Some microbes bind E-cadherin on colonic epithelial cells, with altered polarity or within a disrupted barrier, and trigger b-catenin activation. Other microbes inject effectors (e.g., CagA or AvrA) that activate b-catenin signaling, resulting in dysregulated cell growth, acquisition of stem cell–like qualities, and loss of cell polarity.

(C) Proinflammatory pathways are engaged upon mucosal barrier breach in an evolving tumor. Loss of boundaries between host and microbe engages pattern recognition receptors and their signaling cascades. Feedforward loops of chronic inflammation mediated by NF-kB and STAT3 signaling fuel carcinogenesis within both transforming and non-neoplastic cells within the tumors

Garrett note three factors. First is the DNA damage due to what bacterial elements release. This area clearly needs improved analysis. Second, b-catenin is a driver of E Cadherin, the protein that ties one cell to another. Break E cadherin and the cells start to migrate. We see this in melanoma, especially in melanoma in situ. Third, the activation of the NF-kB pathway, as we have discussed extensively before, is a major driver for proliferation and metastasis.

As Schwabe and Jobin note:

Microbiota and host form a complex 'super-organism' in which symbiotic relationships confer benefits to the host in many key aspects of life. However, defects in the regulatory circuits of the host that control bacterial sensing and homeostasis, or alterations of the microbiome, through environmental changes (infection, diet or lifestyle), may disturb this symbiotic relationship and promote disease. Increasing evidence indicates a key role for the bacterial microbiota in carcinogenesis.

In this Opinion article, we discuss links between the bacterial microbiota and cancer, with a particular focus on immune responses, dysbiosis, genotoxicity, metabolism and strategies to target the microbiome for cancer prevention.

The metaphor as a "super organism" may be apt. The question however may be; as humans change their consumption of or exposure to other organisms or substances that enable or suppress existing organisms, does the homeostatic balance we would expect get disturbed to result in a malignancy. Simply, we now know that cigarette smoking can lead to lung cancer, and transmission of papilloma virus leads to cervical cancers. Is this a result of such disturbances?

As Sfanos et al note:

Chronic inflammation promotes the development of several types of solid cancers and might contribute to prostate carcinogenesis. This hypothesis partly originates in the frequent observation of inflammatory cells in the prostate microenvironment of adult men. Inflammation is associated with putative prostate cancer precursor lesions, termed proliferative inflammatory atrophy. Inflammation might drive prostate carcinogenesis via oxidative stress and generation of reactive oxygen species that induce mutagenesis. Additionally, inflammatory stress might cause epigenetic alterations that promote neoplastic transformation. Proliferative inflammatory atrophy is enriched for proliferative luminal epithelial cells of intermediate phenotype that might be prone to genomic alterations leading to prostatic intraepithelial neoplasia and prostate cancer.

Studies in animals suggest that inflammatory changes in the prostate microenvironment contribute to reprogramming of prostate epithelial cells, a possible step in tumour initiation. Prostatic infection, concurrent with epithelial barrier disruption, might be a key driver of an inflammatory microenvironment; the discovery of a urinary microbiome indicates a potential source of frequent exposure of the prostate to a diverse number of microorganisms. Hence, current evidence suggests that inflammation and atrophy are involved in prostate carcinogenesis and suggests a role for the microbiome in establishing an inflammatory prostate microenvironment that might promote prostate cancer development and progression.

Now with some of the recent immunotherapy drugs the biome can be enhanced. As Leslie notes:

This team pinpointed members of the genus Bifidobacterium as an immune helper: Feeding mice a probiotic that contains several Bifidobacterium species increased the efficiency of a PD-L1–blocking antibody against tumors. The fact that the two teams implicated different bacterial groups doesn’t worry microimmunologist Christian Jobin of the University of Florida College of Medicine in Gainesville. “Different drugs, different bugs, but the same endpoint,” he says. Exactly how the microbiome bolsters the drugs remains unclear. Still, the discovery “opens up novel ways to potentially augment therapy,” says Cynthia Sears, an infectious disease specialist at Johns Hopkins School of Medicine in Baltimore, Maryland. Doctors could, for example, try to beef up antitumor responses with probiotics, although Zitvogel notes that regulatory agencies haven’t approved their use for cancer patients

From Fulbright et al we have the following list of cancer related microbiome elements:

Intestinal bacteria	*Bacterial mechanism*	*Hallmark affected*
enterotoxigenic *Bacteroides fragilis* (ETBF)	*B. fragilis* toxin (BFT)	sustaining proliferative signaling
		genome instability and mutations
	unknown mechanism	tumor-promoting inflammation
Fusobacterium nucleatum	FadA adhesin	sustaining proliferative signaling
	Fap2 adhesin	avoiding immune destruction
pks+ Escherichia coli	colibactin	genome instability and mutations
		sustaining proliferative signaling
Enterococcus faecalis	unknown mechanism	genome instability and mutations
Alistipes spp.	unknown mechanism	tumor-promoting inflammation
Bifidobacterium spp.	unknown mechanism	inhibits avoiding immune destruction
Bacteroides thetaiotamicron and *B. fragilis*	unknown mechanism	inhibits avoiding immune destruction

The authors continue:

Normal tissues tightly regulate growth-promoting and death-inducing signals to maintain homeostatic cell densities, tissue architecture, and function. Dysregulation of these signaling pathways can lead to sustained cellular proliferation. The intercellular adhesion molecule, E-cadherin, is a common target engaged by intestinal bacteria that promotes epithelial proliferation by activating the Wnt/û-catenin pathway. For example, enterotoxigenic Bacteroides fragilis (ETBF), resident among the microbiota of some individuals, secretes B. fragilis toxin (BFT) that promotes cleavage of E-cadherin.

This enables the nuclear translocation of û-catenin, subsequent transcription of proto-oncogene c-Myc, and colonic epithelial hyperplasia. Through a similar mechanism, Fusobacterium nucleatum enhances epithelial proliferation through engagement of its adhesin FadA with E-cadherin. Neutralizing FadA abrogated the tumor-promoting activities of F. nucleatum in a murine xenograft cancer model, demonstrating the potential of targeting bacterial interactions with E-cadherin as a novel strategy in mitigating cancer progression.

Taken together, these studies demonstrate that the microbiota can be a source of activating signals for aberrant epithelial proliferation as an initiating step in cancer development.

The change in e-cadherin is critical. This is a binding protein and when broken the cell now has the ability to move about and this in many cancers is the first step towards an aggressive growth pattern.

From Vogtmann and Goedert they indicate the following possible list:

Pathogen	*Cancer Type/Organ*
H pylori	Gastric
H pylori	Hepatobilliary
Salmonella typhi	Hepatobiliary
Neisseria elongata	Pancreas
Streptococcus mittis	Pancreas
Porphyromonas gingivalis[66]	Pancreas
Mycobacterium tuberculosis	Lung
Spirochaetae	Lung
Bacteroides	Lung
Synergisters	Lung
Fusobacterium	Colorectal
Porphyromonas	Colorectal
Borellia burdorfii	Cutaneous B Cell Non Hodgkins Lymphoma
Chlamydophilia psittaci	MALT Lymphoma

These types of lists have been developed by many authors. Generally they lead to many similar and identical pathogens but frequently to an added new set.

14.1.4.3 Obesity

We have discussed elsewhere that obesity is directly and as a result of its inflammatory nature is a putative cause of cancer. Obesity is a major epidemic throughout the world. It is insidious in that its effects are slow to develop and then once started are often near impossible to stop. It is a pandemic of a chronic and debilitating state, with disease sequellae. As Arnold et al note:

Worldwide, we estimated that 481,000 or 3·6% of all new cancer cases in 2012 were attributable to excess BMI. PAFs were greater in women compared with men (5·4% versus 1·9%). The burden was concentrated in countries with very high and high human development index (HDI, PAF: 5·3% and 4·8%) compared with countries with moderate and low HDI (PAF: 1·6% and 1·0%).

Corpus uteri, post-menopausal breast and colon cancers accounted for approximately two-thirds (64%) of excess BMI attributable cancers. One fourth (~118,000) of all cases related to excess BMI in 2012 could be attributed to the rising BMI since 1982.

[66] See Thaiss et al, "*The microbiome and innate immune system also cooperate in the eradication of bacterial infection. Sometimes, neither innate immunity nor colonization resistance is sufficient to ensure the expulsion of pathogens. Instead, a combination of the two is required, as in the case of cooperation in the host defence against Citrobacter rodentium, a bacterium that can cause disease in mice. However, such combinatorial responses can be subverted by the pathogen. During infection with Salmonella Typhimurium, microbiota-induced IL-22 elicits a response that targets commensal bacteria and liberates a colonization niche for the pathogenic bacterium118. Porphyromonas gingivalis, an oral bacterium that is associated with periodontitis, evades the host by modulating the TLR2 pathway to support a niche for dysbiosis and subsequent inflammation.*"

Obesity has a massive amount of secondary effects. It generates a feeding ground for many microorganisms, provides nutrients, creates a multiplicity of reactive oxygen species, and the like.

14.1.5 Biomes

We now examine two rather extreme cases. First the oral biome which we know is a complex and active biome. There has been extensive study of the relationship between that biome and head and neck cancers, including oral cancers. The lack of long term stability of the biome is often a problem in examining it for microorganism effects. The second is the bladder, an organ which has been generally assumed to be sterile. In reality there is a complex biome of microorganisms present but they generally cannot be studied using more classic techniques. We have one area of complex well known microorganisms and another presenting a new territory to explore.

We present two examples of these biomes. One is obvious, the oral cavity. The second is counter intuitive based on classic medical training, namely the bladder. We all assumed the mouth to be filled with bacteria. In fact we often wonder how the body manages to battle against this excess. In contrast we all assume urine is sterile. In fact there are commensurate bacteria in the bladder. These we discuss.

14.1.5.1 Oral

Lin et al have examined the oral biome. The following Table is a brief summary of the microbiome and this may very well be incomplete. Furthermore it may change from person to person and even with a single person there may be substantial temporal changes.

Region	Microorganism
Tooth Surface	Streptococcus mutans, Actinomyces, Eubacterium, Peptostrepptococcus
Tonsil	Streptococcus viridans, Neisseria species, — Haemophilus influenzae, coagulase-negative Staphylococci
Tongue	Veillonella atypica, Porphyronas gingiva I is, Selenomonas species, Actinobacillus actinomycetemcomitans, Prevotella intermedia, Capnocytophaga species, Streptococcus faecalis, Eikenella corrodens
Gingival Surface	Fusobacterium, Prevote I la, Porphyromonas
Oropharyngeal region	Streptococcus salivarius, Streptococcus mutans, Streptococcus anginosus, Streptococcus pyogenes, Streptococcus pneumoniae, Haemophilus influenza, Haemophilus parainfluenzae
Dental Plaque	Actinomyces, Rothia, Kocuria, Arsenicicoccus, Microbacterium, Propionibacterium, Mycobacterium, Dietzia, Turicella, Corynebacterium, Bifidobacterium, Scardovia, Parascardovia

Lin et al then note:

Oral microbiome, by definition, is the collective genomes of microorganisms that reside in the oral cavity. Many researchers believe that the characterisation of oral microbiome is an essential step in understanding oral health and systemic diseases. The oral cavity has densely populated microbial communities and has the largest core of commonly shared microbes among unrelated individuals. As such, oral microbiome provides an ideal source for biomarker discoveries due to low inter- and intra- biological variations, in contrast to other tumour biomarkers originating from the host.

The oral cavity and associated nasopharyngeal regions are also an ideal environment for the growth of microorganisms. The average normal oral temperature is 37°C without significant fluctuation, providing bacteria a stable habitat to thrive. In addition, saliva maintains a stable pH of 6.5 to 7.5, the preferred pH for most bacteria species. Saliva also keeps bacteria hydrated and acts as a medium to facilitate the transportation of nutrients to microorganisms. As such, the

oral cavity harbors more than 700 bacterial species and is one of the most densely populated anatomical sites within the human body…

Studies have established that chronic inflammation is responsible for 25% of human malignancies and represents the seventh hallmark in the development of cancers. Chronic inflammatory mediators cause or facilitate increased cell proliferation, mutagenesis, oncogene activation, and angiogenesis that ultimately lead to the loss of normal growth control and cancer.

Bacterial infection is one of the major causes of chronic inflammation. The strongest link established between bacterial infection and the development of cancer due to chronic inflammation to date is the association between Helicobacter pylori (H. pylori) and adenocarcinoma of the stomach, while other known associations include Salmonella typhi and gallbladder cancer, Streptococcus bovis and colon cancer, Chlamydia pneumonia and lung cancer, and Bartonella species and vascular tumour formation. In general, studies have shown that bacteria alone are unable to induce cancer; the process is commonly accompanied by chronic inflammation and requires independent mutations in oncogenic signalling pathways

The study carried out by Schmidt et al., (2014) investigated the oral microbiome of five oral cancer patients and eight oral pre-cancer patients using 16s rRNA gene amplicon next-generation sequencing.

The biospecimens were collected using swabs on the oral lesion and a contralateral normal site. This study reported a significant decrease in abundance of Firmicutes and Actinobacteria in cancer patients. A significant decrease in these phyla were also confirmed in pre-cancer patients, suggesting that oral lesion-associated shifts in oral microbiome may occur early in oral cancer development and/or herald cancer progression.

The study from Guerrero-Preston et al., (2016) utilised oral rinse as biospecimens. The oral microbiome of 19 HNSCC patients and 25 normal healthy individuals were investigated using 16s rRNA gene amplicon next-generation sequencing and a decrease in microbial richness and diversity was reported in cancers. The enriched presence of Lactobacillus or the loss of Haemopilus, Neisseria, Gemellaceae or Aggregatibacter in saliva was reported as a potential biomarker for HNSCC. While HPV status did not have a significant impact on the oral microbiome, it is speculated that the small sample size may have influenced the outcomes.

The findings from both studies indicated that microbial diversity and taxonomic composition of the oral microbiome may be useful biomarkers for HNSCC as well as provide a solid framework for future oral microbiome research.

Thus the oral cavity may be an interesting area to examine for the development of microorganism based malignancies. However its extreme uniqueness and instability would make such an examination quite difficult.

14.1.5.2 Bladder

The bladder has for a long time been considered a sterile environment. It is often in contradistinction to the oral cavity. However recently its biome has been examined and is beginning to be ascertained. As noted in Ainsworth:

The dogma that urine, and by extension the bladder, must be sterile to be healthy has been overturned, and microbes are being discovered throughout the urinary system. Researchers are investigating potential roles for them in healthy bladders and in a range of conditions, including urge incontinence — where people experience a sudden need to urinate — and in some cancers. Burton's team has found traces of bacteria in cancerous kidneys, for example. Although still at the early discovery stage, research into the bladder's microbes promises to transform understanding of the urinary tract. "It's really grown and exploded rapidly," says Burton…

The potential link with chronic inflammation raises the question of whether repeated urinary tract infections might be involved in the development of bladder cancer. One of the largest epidemiological studies of bladder cancer conducted so far reported4 in 2015 that repeated, regular bouts of cystitis were associated with increased risk, but whether the association was causative is unclear.

Further studies will be needed to confirm any links, which remain "a little tenuous" at the moment, according to Burton. …., for example, a team in Japan reported5 that people with bladder cancer who drank a probiotic containing Lactobacillus casei (sold commercially as Yakult), while also receiving chemotherapy treatments infused into the bladder, had recurrence rates that were 15% lower than those of subjects receiving chemotherapy alone. Critics of the study said that the pattern of patient dropout and lack of blinding may have undermined its conclusions, although the authors disagreed.

Previous studies in animals, conducted by several research groups, also suggest that probiotics can have anticancer effects in the bladder. These studies suggest that probiotics deserve further investigation, says Burton.

Thus the biome of the bladder may have any combination of neutral, negative or positive effect on the potential for malignancies. In contrast to the oral cavity, the microorganisms in the bladder are complex and require sophisticated techniques to determine.

14.1.6 Therapeutics

Cancer therapeutics, especially the explosive use of immunotherapy, has demonstrated an ability to attack cancer cells as one would attack any foreign body using the elements of the immune system. Many of the immune approaches use T cell methods and even uniquely targeted T cells developed using chimeric approaches. However, it must be remembered that the immune system has a plethora of attack mechanisms. In particular the innate immune system has a powerful set of near real time attack cells and molecules that can recognize an aberrant cell or collection thereof and commence its elimination. Equally, as we have discussed, the microbiome can enhance that effect. It likewise can, if improper, be the actual cause of the malignancy.

In the paper by Thaiss et al they note:

The influence of the microbiota on innate immunity has been shown to affect the host response to cancer therapy. For example, germ-free mice and mice that are treated with antibiotics both show a diminished response to immunotherapy by CpG oligonucleotides and chemotherapy owing to the impaired function of myeloid-derived cells in the tumour microenvironment58. Furthermore, commensal Bifidobacterium enhances immunity to tumours through antibodies directed against programmed cell death 1 ligand 1 (PD-L1) through the augmentation of dendritic-cell function.

These studies might open up a fascinating avenue of research to prevent cancer and develop cancer therapeutics through manipulation of the microbiota.

Thus it is now well known that there is a strong linkage between the microbiome and cancer therapeutics as well.

14.1.6.1 Putative Microbial Therapeutics

The following list is one which reflects those with some putative efficacy in humans (see Zitvogel et al):

Bacterial species[67]	*Cancer type*	*Interventions and outcomes*
Streptococcus pyogenes* and *Serratia marcescens	Osteosarcoma	Coley's toxins: injection of *S. pyogenes* and *S. marcescens* in patients with sarcoma, with some evidence of objective response
***Mycobacterium bovis* BCG**	Urothelial superficial cancers	Intravesical treatment of a live attenuated form of *M. bovis* reduces the risk of short- and long-term relapse
***Lactobacillus casei* str. Shirota (found in the fermented milk product Yakult)**	Superficial bladder cancer	Immune-mediated effects (by NK cells and macrophages) and decreased tumour recurrence (except with multiple secondary tumours)
IMM-101 (heat-killed *Mycobacterium obuense*; NCTC 13365) with gemcitabine	Melanoma and advanced pancreatic ductal adenocarcinoma	Activation of APCs, granulocytes and γδ T cells. Increased survival in metastatic disease in a randomized phase II trial
Live-attenuated *Listeria monocytogenes* expressing mesothelin *(*CRS-207*)* with GVAX-cyclophosphamide	Advanced pancreatic ductal adenocarcinoma	Priming of mesothelin-specific CTLs, loss of regulatory T cells and tertiary lymphoid organ formation, and increased overall survival
IL-13–PE: recombinant cytotoxin consisting of human IL-13 and PE	Adrenocortical carcinoma	Majority of patients produce neutralizing antibodies against IL-13–PE within 2–3 weeks
IL-4–PE: chimeric fusion protein composed of IL-4 and PE	Astrocytoma	Phase I trial: no systemic complications, median survival of 8.2 months and evidence of necrosis on MRI scans in several patients
Attenuated strain of *Salmonella enterica* subsp. *enterica* serovar Typhimurium: VNP20009	Metastatic melanoma and refractory solid tumours	Phase I trial of intravenous infusion of *S.* Typhimurium led to inflammation, DC and T cell activation and evidence of bacterial tumour colonization; however, there was no tumour regression
TAPET-CD: an attenuated *Salmonella* bacterium that expresses the *Escherichia coli* cytosine deaminase gene	Head and neck squamous cell carcinoma or adenocarcinoma of the oesophagus	Evidence of bacterial colonization and confirmation of the conversion of 5-FC to 5-FU in 2 out of 3 tumours
Genetically modified *Corynebacterium diphtheriae*: Tf-CRM107 is a conjugate of transferrin and a point mutant of diphtheria toxin	Malignant brain tumour	MRI scans showed regression of tumour volume in 9 out of 15 patients with no evidence of severe local or systemic complications at low dose

Now the following list is one of putative efficacy yet to be fully vetted in humans (Zitvogel et al):

[67] Bacteria that have putative anticancer properties in humans, Zitvogel et al

Bacterial species[68]	*Cancer type*	*Interventions and biological effects*
Clostridium novyi **(*C. novyi* non-toxic strain spores)**	Orthotopic F98 rat glioma and dogs with spontaneous solid tumours	Intratumoural injections led to tumour haemorrhagic necrosis, lysis and regression
Lactobacillus casei	Orthotopic and transplantable bladder tumours and their metastases	Oral or intravesical injection of dead or alive bacteria increased the levels of IFNγ and the recruitment of neutrophils
Lactobacillus rhamnosus **GG**	Bladder tumours	Weekly intravesical instillations directed chemokine and/or cytokine release, recruitment of NK cells and direct cytotoxic effects on cell lines *ex vivo*
Alistipes shahii	MC38 colon cancer	Gavage after antibiotic treatment increased the production of TNF by intratumoural myeloid cells
Bacteroides fragilis **and *Burkholderia cepacia***	MCA205 sarcomas and MC38 and CT26 colon cancers	Oral gavage of *B. fragilis* stimulated the production of IL-12 by bone marrow-derived DCs *in vitro.* The mechanism of *B. cepacia* remains unknown
Prevotella **spp. and *Oscillibacter* spp.**	Subcutaneous hepatocellular carcinoma	Oral administration of Prohep, a probiotic mixture, altered the microbiota and reduced tumour growth
Enterococcus hirae **and *Barnesiella intestinihominis***	Sarcoma	Bacterial translocation: induction of TH1 cells and pathogenic TH17 cells, intratumoural regulation of Treg cells and IFNγ-producing γδ T cells, respectively
Bifidobacterium longum **and *Bifidobacterium breve***	Melanoma	Oral gavage led to the activation of DCs and an increased frequency of tumour-specific CTLs
Lactobacillus casei **str. Shirota**	MCA induced cancer	*L. casei* str. Shirota mixed into mouse diet delayed carcinogenesis through enhancement of NK cell cytotoxicity
Lactobacillus casei **ATCC334**	Colon cancer SW620 cells (Caco2 *in vitro*)	Secretion of ferrichrome, which induces JNK-associated induction of DNA damage-inducible transcript 3. Enhanced apoptosis of colon cancer cells
Lactobacillus casei **BL23**	DMH-associated colorectal cancer	Oral administration of *L. casei* BL23 led to differentiation of T cells towards a TH17-biased immune response (with the secretion of IL-6, IL-17, IL-10 and TGFβ)
Lactobacillus acidophilus	CRC *ApcMin*/+	Daily administration of yogurt formulation decreased overall intestinal inflammation
Bifidobacterium lactis **and RS**	Colorectal rat-azoxymethane model	The addition of RS to the diet andbacteria induced apoptosis in tumour cells at the time of cancer initiation
Antibiotic-induced loss of members of the Firmicutes and Bacteroidetes phyla; gain of members of the Proteobacteria	LLC and B16F10 lung metastases	Microbiota modifications following antibiotic treatment induced the loss of γδ T cells producing IL-17A

[68] Bacteria that have putative anticancer properties in experimental models, Zitvogel et al

Bacterial species[68]	*Cancer type*	*Interventions and biological effects*
***Bacillus polyfermenticus* and its culture medium**	HT-29, DLD-1, Caco2 human colon cancer in mice	Cyclin D1 expression required for ErbB-dependent cell transformation was decreased by culture medium injections near the tumour sites
Propionibacterium freudenreichii	Human colon adenocarcinoma HT-29 cells	Production of SCFAs, which induced pH-dependent differential cell death processes
L. acidophilus* and *L. casei	LS513 colorectal cancer cell line	Sensitization of colorectal cancer cells to 5-FU-induced apoptosis
***Enterococcus faecium* RM11 and *Lactobacillus fermentum* RM28**	Caco2 cell lines	Antiproliferative effects on CRC cells
***Lactobacillus delbrueckii* CUI22**	HT-29 cell line; probiotic supernatant	Apoptosis and necrosis through the production of bacterial hydrogen peroxide and superoxide radicals
***L. acidophilus* 606**	HT-29 colon cancer line	Cell-bound exopolysaccharides induced the activation of autophagic cell death promoted directly by the induction of beclin 1 and GRP78
***B. lactis* Bb12 and *L. rhamnosus* GG**	Caco2 cancer cell line	Induced apoptosis through the mitochondrial route
L. acidophilus* and *L. casei	LS513 colorectal cancer cell line	Sensitized colorectal cancer cells to 5-FU-induced apoptosis

14.1.6.2 Mechanisms of Microbials

We now briefly examine the mechanisms which may be the basis for the therapeutic efficacy.

From Zitvogel et al:

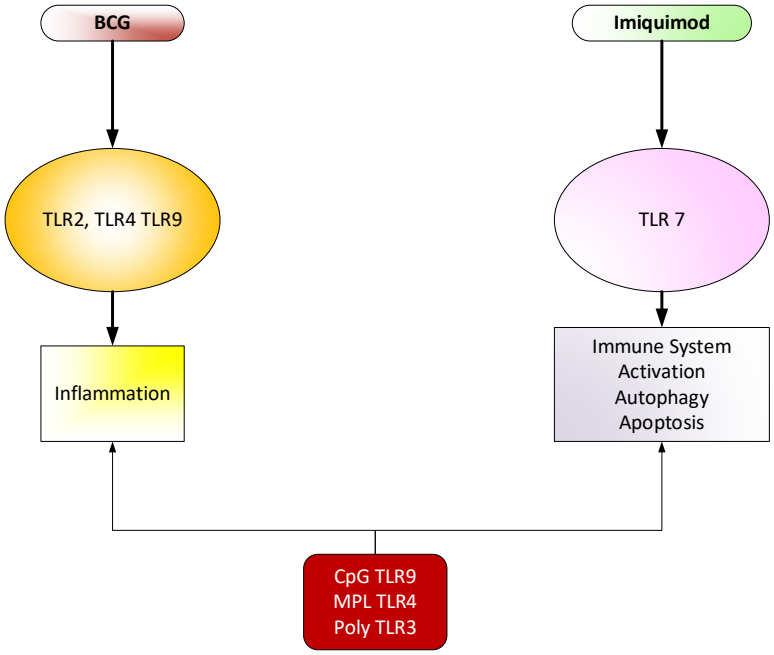
Bacteria produce various molecules that may affect the survival and growth of cancer cells, or that modulate anticancer immunosurveillance. These include bacterial toxins that have direct anticancer properties, ligands of PRRs that affect the Immune response and metabolites that affect host metabolism. There is no clear distinction between the latter two categories, as some metabolites can act on PRRs; this has been demonstrated for phenazines from Pseudomonas aeruginosa and phthiocol from Mycobacterium tuberculosis, which act on aryl hydrocarbon receptor (a PRR that functions as a transcription factor) and for N-acetylglucosamine (a sugar subunit of bacterial peptidoglycan), which acts on the hexokinase PRR to activate inflammation.

The authors procced to detail three specific mechanisms as follows:

1. Bacterial toxins. Bacteria produce different toxins and antibiotics, which allow them to compete with other microorganisms. Bacterial toxins may have direct anticancer effects, as illustrated for anthracyclines produced by Streptomyces spp. Indeed, anthracyclines, including doxorubicin, are widely used in anticancer chemotherapy and can induce immunogenic cell death, thereby stimulating anticancer immune responses78. However, it remains to be determined whether toxins are produced by intestinal bacteria at doses high enough to mediate such anticancer effects….

2. Ligands of PRRs. PRRs mostly recognize pathogen-associated molecular patterns (PAMPs), although they may also have endogenous ligands. One well-known PAMP is bacterial lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, which interacts with TLR4. LPS can stimulate inflammatory responses when bacteria enter the systemic circulation through breaches in the intestinal barrier. This can occur after cancer treatment with radiation therapy, and may improve the inhibition of tumour growth by activating T cells82. TLR4 is also thought to be fundamental for the anticancer effects of BCG83. PAMPs can be used as vaccine adjuvants to elicit an immune response against viruses that can cause cancer…..

We demonstrate this below:



It should be noted that TLRs, Toll Like Receptors have been shown clinically to be quite effective. TLR7 is a strong effector on antiviral activity. There are TLRs for many targets and when we combine these with specific microorganisms targets at cancer cells, we get a power set of immune tools in the innate immune system.

3. Bacterial metabolites. The microbiota has a key role in human metabolism; approximately 50% of metabolites in the plasma are estimated to have a bacterial origin. The gut microbiome synthesizes all SCFAs and secondary bile acids, polyamines and vitamins. Bacterial metabolites may affect cancer development and the efficacy of antineoplastic therapies.

14.1.7 Observations

The microbiome is now recognized as an essential element of homeostasis. Its changes are also recognized as putative causes of disease and specifically malignant changes. Furthermore the modulation of the microbiome may very well present opportunities to mitigate against various

malignancies both directly as well as through secondary means. This paper is not intended to be a definitive statement on the efficacy of the microbiome as an adjunct in cancer care. Its intent is solely to attempt to identify the issue and lay forth several pathways for investigation. All too often the microbiome is not even recognized as an element of a balanced scale of health.

14.1.8 Status of the Microbiome

As Garrett noted:

Microbiota studies in cancer remain at an early stage. Information gathering and descriptive studies are still necessary, and many critical questions remain. What other mechanisms might microbes use to influence tumorigenesis?

If single microbes can compromise antitumor immunity or enhance susceptibility to oncomicrobes, are there configurations of the microbiota that do this, too (or are protective)? Are there microbes or microbiotas that enhance responsiveness to immunotherapies or other therapeutic interventions? To answer these questions, it is important to identify the key next steps in understanding how the human microbiota affects tumor growth and spread.

The understanding of the microbiome as part of the immune system and in terms of cancer mitigation is just beginning to be explored. The main challenges are twofold.

First is necessary to have the tools to be able to explore the consequences of the interaction of the microbiome and the normal cell.

Second, is the challenge of dealing with a temporally and spatially varying microbiome. This can be a real challenge. It presents such a complex environment that the modelling tools are far from adequate.

14.1.9 Classic Carcinogenesis vs Microbiome Modulation

As Vogtmann and Goedert conclude

There is epidemiologic evidence for associations between the human microbiome and cancer, particularly gastric and colorectal cancer. However, epidemiologic studies of this association have thus far been very limited, typically with small sample sizes and cross-sectional designs with single-time sampling. Although case–control studies can provide initial insights into microbial associations with cancer, reverse causation is of great concern.

In a case–control study, it is not possible to determine whether the carcinogenic process changes the local environment and creates a new niche for microbes or whether alterations in the microbial population or its functions contribute to carcinogenesis. New studies that incorporate repeated, prospectively collected oral, faecal, tissue, and other samples will be important to elucidate the temporal nature of microbial associations with cancer. Future studies should also incorporate the study of fungi, protists, and viruses, in addition to bacteria and archaea, to fully characterise the human microbiome and its relationship with cancer risk. In addition,

standardised methods for the collection of samples, preparation and handling of samples, and bioinformatic processing of data are needed and work is ongoing in this area (e.g., www.mbqc.org). …

Finally, there is a need to explore postulated microbe-mediated carcinogenic mechanisms through transcriptomics, proteomics, metabolomics, and novel immunologic assays. Microbiome associations with cancer may differ across many host factors, including sex, age, smoking, alcohol consumption, diet, obesity, physical inactivity, and polymorphisms in major human oncogenes. Explicit consideration of these host factors may yield clear stratification of microbiome associations with the various malignancies.

Ultimately, across the identified strata, microbiome associations should be translated into practical applications in order to accelerate the diagnosis of cancer or precancer, to increase efficacy and reduce toxicity of cancer therapy… and ideally to prevent cancer by interrupting a microbial carcinogenic pathway.

Microbiome modulation as discussed above is a challenge in ascertaining causal relationships. As we discussed previously this challenge is drastically different from a normal causal relationship we normally attempt to define.

14.1.10Microbiome within Microbiome

There is now another layer to this complex environment. Namely the interaction of phages, viruses, with bacteria and then in the microbiome, As Guglielmi has noted:

Though where the viruses end up is unclear, those data and other recent studies have scientists wondering whether a sea of phages within the body—a "phageome"—might influence our physiology, perhaps by regulating our immune systems. "Basic biology teaching says that phages don't interact with eukaryotic cells," says phage researcher Jeremy Barr of Monash University in Melbourne, Australia, who led the study published this week in mBio. He's now convinced "that's complete BS." For decades, most medical research on phages focused on turning these bacterial parasites into antibiotics.

There have been some compelling success stories, but phage therapy has struggled to become a dependable treatment. Yet Barr's earlier research showed that phages might naturally help protect us from pathogens. Studying animals ranging from corals to humans, he found that phages are more than four times as abundant in mucus layers, like the ones that protect our gums and gut, as they are in the adjacent environment. The protein shell of a phage, it turned out, can bind mucins, large secreted molecules that together with water make up mucus. This works out well for both phages and mucus making animals. Sticking to mucus enables the phages to encounter more of their bacterial prey. And as a result, Barr showed in a series of in vitro studies, the viruses protect the underlying cells from potential bacteria pathogens, providing an additional layer of immunity.

This it is possible to use a complex sat of the microbiome elements, one against the other, in the world of microbiome therapeutics.

It is well know that inflammation is related to a multiplicity of cancers. Further many cancers are now treated by various immunotherapeutic means. In this note we examine inflammation in more detail and then focus on the actions of the innate immune system as both a factor in certain inflammatory cancers as well as a means to combat these cancers. The focus on the immune system is on the innate side, since this generally is the side where the fastest response is. This is in contrast to the significant progress on the use of the adaptive elements and the addition of T cell elements such as CAR-T cells or the use of Mabs for blocking such as PD-1 and T cell attacks.

We will focus this paper around a paper by Stallone et al. The authors note:

Pentraxin-3 (PTX3) is a member of the pentraxin family of innate immune regulators which includes C-reactive protein (CRP). PTX3 has been implicated in angiogenesis, proliferation and immune escape in cancer. In the present study, we evaluated PTX3 tissue expression and serum concentration as a biomarker to discriminate prostatic inflammation and benign prostatic hyperplasia (BPH) from prostate cancer (PCa), and to determine whether PTX3 status may predict progression from BPH to PCa. …We found reduced PTX3 tissue expression in patients with prostatic inflammation/BPH compared to patients who developed PCa.

In the latter group, there was an increase in PTX3 tissue expression between the first and second prostate biopsy. PTX3 serum levels were also higher in patients with PCa than in patients with inflammation/BPH. In contrast, there was no difference in serum PSA or CRP levels in these two groups. ROC curve analysis confirmed the reliability of PTX3 serum levels in predicting PCa development, identifying a cut-off value of 3.25 ng/ml with a sensitivity and a specificity of 89.3 and 88.5%, respectively. In summary, our results encourage further evaluation of PTX3 as a tissue biopsy and blood-borne biomarker to discriminate BPH from PCa.

We chose this as a reference point because it starts the process of examining several key factors:

1. PSA has taken a continual beating for its lack of specificity and sensitivity. Although it has managed to survive the endless assault by Government entities, it does have weaknesses.

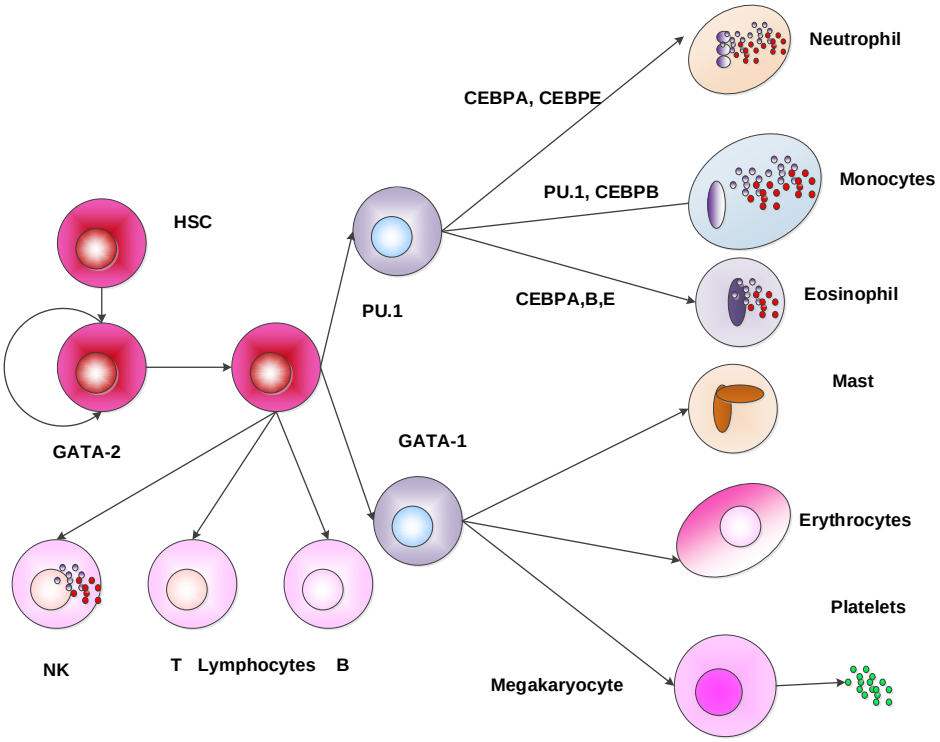
2. BPH is generally accepted to be an inflammatory response and there is also the understanding that PCa is likewise. Where one sees progression from inflammation to malignancy, this is not always inevitable or even justified. For example, High Grade PIN has been assumed to be a progenitor of PCa. Yet we have shown that it can easily disappear and not turn into PCa in a reasonable time period, say ten years. Thus inflammation is suspect but not dispositive to cancers.

3. This referred to work focuses on the innate immune system, especially the humoral leg, and specifically pentraxins. Thus the work opens the windows on elements for which we have seen relatively little work.

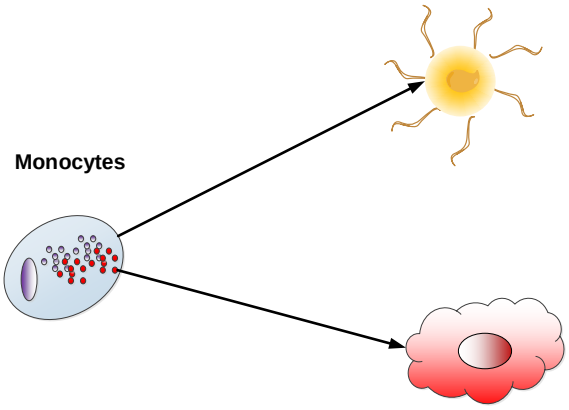
4. Finally, in examining inflammation and the innate system as a cause, we also examine it as a tool to address a multiplicity of cancers.

Yet we have also included a brief discussion on the bromo domain epigenetic factors which demonstrate another inflammatory element in malignant initiation.

We start with a simple review of the primary blood cells and do this to set up a basis for the cell based elements in the innate system. In the adaptive system we have principally the B and T cells. They utilize the antigen presenting capabilities of the other cells and then via the B cells ability to deliver antibodies can then go on the attack using a variety of chemical drivers. In contrast the innate cells, those in the cellular part of the innate immune system go on the hunt alone. Such cells as NK cells, neutrophils and dendritic cells. We have seen the dendritic cells used in certain cancers such as prostate and the NK cells used in what is called the cytokine induced killer cells, CIK, in MDS, a hematological malignancy. Thus there is a developing cellular set of tools available. In contrast we also have the humoral part of the innate system, with such elements as the complement system, pattern recognition receptors and pentraxins.



Monocytes themselves subdivide into dendritic cells and macrophages, which are like "hunter-gatherers" working the human body and bringing back the antigens they find.



14.2 INFLAMMATION

Inflammation is the response of various cells to the presence of some factor which is deemed foreign and putatively a threat. As we will note, the human immune system responds in both the innate and adaptive manner to any perceived threat by a plethora of means. It sends out chemicals to attack the perceived invader and ultimately tries to identify the invader, kill it off, while remembering what it looked like so that the next time it can respond more quickly. Sometimes it works and other times it does not. But simply stated inflammation is the response of the immune system in some manner to something which activates it. This may sound a bit like circular reasoning by defining the process by its very existence, but that is simply what it is.

The problem with inflammation is that it can also cause more problems than what it solves. For example, H pylori can cause an inflammatory response in the stomach and the consequence is a MALT neoplasia, a cancer, resulting from this prolonged inflammatory response. Inflammatory responses release a variety of molecules whose goal is ultimately to rid the body of the invader. However, if a chronic situation is created where the invader is subdued but persists, the immune system can be keep in an "on" state resulting in the lasting presence of the secretions meant to kill off the invader. These powerful substances can then result in the activation or repression of homeostatic pathways resulting in the development and spread of neoplasia.[69]

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[69] From Doan, Immunology 2nd Ed, Lippincott (New York), 2013

A. Cytokines: Low-molecular-weight soluble protein messengers that are involved in all aspects of the innate and adaptive immune response, including cellular growth and differentiation, inflammation, and repair. Originally called lymphokines and monokines to reject lymphocytic or monocytic origin, we now recognize that these substances are produced by a wide variety of leukocytes and non-leukocytes. A large number of cytokines have been identified, although the roles of many of them are not yet fully understood. Many cytokines are crucial in regulating lymphocyte development and in determining the types of immune responses evoked by specific responses.

B. Chemokines: Low-molecular-weight cytokines known as chemokines (chemoattractant cytokines) stimulate leukocyte movement. Leukocytes are guided by chemokine concentration gradients to the site of an infection or inflammation (a process called homing). They are divided into four types based on the presence of certain structural motifs involving the numbers and intervals between cystine residues: C, CC, CXC and CX3C.

C. Adhesion molecules Often, leukocytes must interact directly to contact other cells under somewhat adverse conditions such as during rapid flow within the circulatory system or under weak ligand-receptor binding.

14.2.1 Inflammation and Cancer

Inflammation may result in the formation of additional cells as well as the release of a variety of signalling elements. This release and activation can often set the path for malignant growth as well. Thus understanding inflammation is essential to understanding the development of many cancers.

As noted by Aggarwal et al:

Cancer is now generally believed to be a preventable disease. Only 5% to 10% of all cancers are caused by inheritance of mutated genes and somatic mutations, whereas the remaining 90% to 95% has been linked to lifestyle factors and environment (1). Almost 30% of all cancers have been attributed to tobacco smoke, 35% to diet, 14% to 20% to obesity, 18% to infections, and 7% to radiation and environmental pollutants. The underlying mechanisms by which these risk factors induce cancer are becoming increasingly evident. One process that seems to be common to all these risk factors is inflammation.

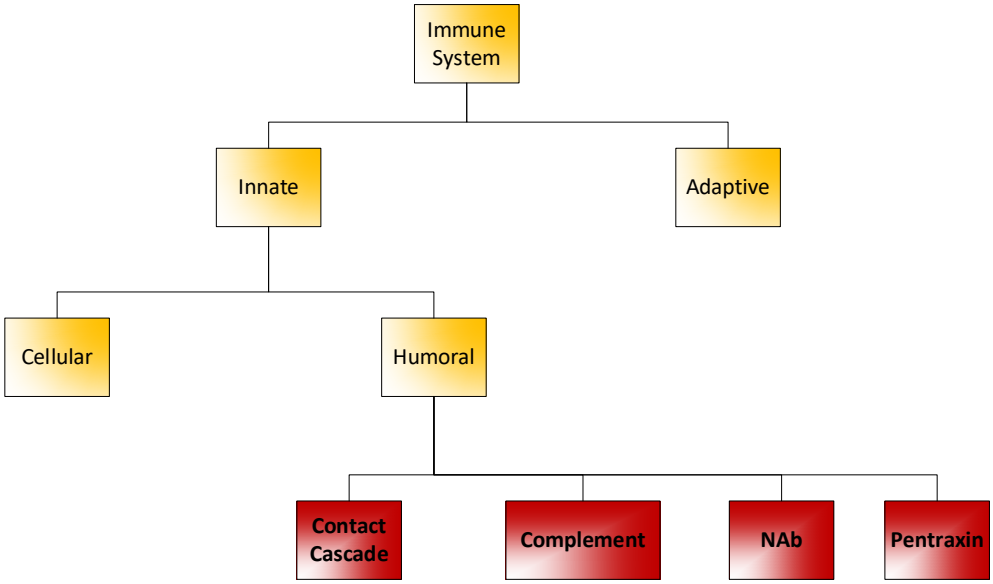
Inflammation is also common whenever cancer is seen or first discovered,

From Kundu et al:

Infection	**Cancer**
Kaposi's sarcoma herpes virus (KSHV)/Human herpes virus-8 (HHV8)	Kaposi's sarcoma
Endometriosis	Endometrial adenocarcinoma
Pelvic inflammatory disease	Ovarian cancer
Barrett's esophagitis	Esophageal cancer
Inflammatory bowel disease	Colorectal cancer
Chronic gastritis (usually with *H. pylori* infection)	Gastric cancer
Infection with Hepatitis virus B and C, hepatic fibrosis	Hepatocellular carcinoma
Telangiectatic features with inflammatory syndrome	Telangiectatic adenoma and hepatic malignancy
Thyroidtis	Papillary thyroid carcinoma
Asbestos	Malignant mesothelioma
Hemophagocytic lymphohistiocytosis (Epstein-Barr virus infection)	T cell lymphoma
Schistosomiasis	Bladder cancer
Primary sclerosing cholangitis	Cholangiocarcinoma
Chronic cholecystitis	Gall bladder carcinoma

Adhesion molecules provide stable cell-to-cell contact necessary for both innate and adaptive immune responses as well as for many other intercellular activities. Although a seemingly simple activity, the ability of cells to examine the surface of other cells and to establish stable contact with them is vital. For cells to communicate and for cell-surface receptors and ligands to interact, the cells must be able to establish and maintain relatively prolonged surface-to-surface contact. Types of adhesion molecules include integrins, selectins, and addressing.

We layout the immune system as shown below. The first partition is on innate and adaptive. The former is fast and non-selective, to a degree, and the second is slower but generally highly selective and with memory. The innate can then be further divided into cellular and then humoral. Cellular innate consists of such cells as macrophages, neutrophils, dendritic cells, natural killer cells, and the like. The humoral system consists of the complement system (classic, alternative, mannose), the contact, the natural anti-body and the pentraxin.



What we will examine is the cycle between:

1. Irritant: Everything starts with something. Philosophically and physically this has a basis in observation. Thus before any inflammation we need a start, usually caused by some irritant. We know the immune system responds to a variety of antigens, whether they be polysaccharides, viral RNA, reactive oxygen species (ROS) or whatever else may induce the action of an immune system element. Thus it may be as complex as a viral infection or as mundane as a dietary complexity resulting in a proliferation of ROS.

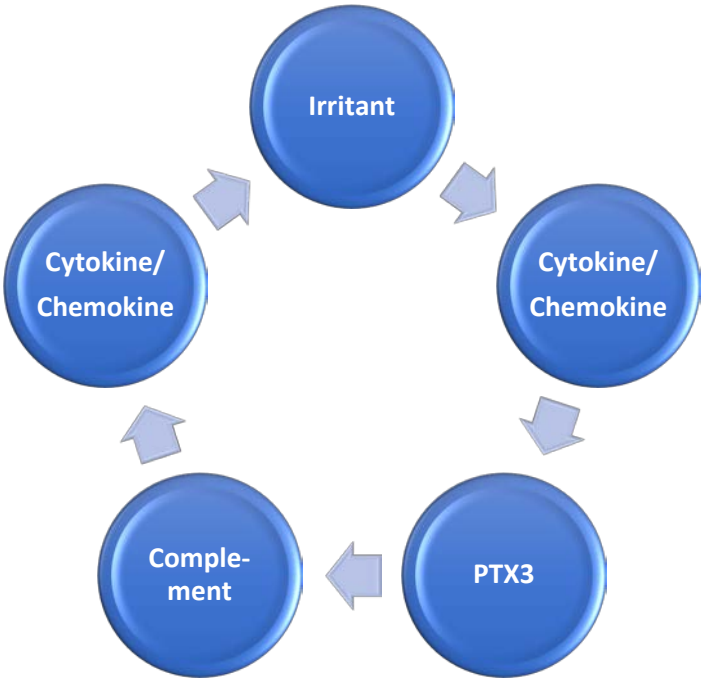
2. Innate System Response: Irritants may cause an immediate type of reaction most likely from the innate system.

3. Inflammation: Inflammation is the interaction of the cell, the irritant, and the elements of the immune system. Cells start responding as well as the immune elements: cells and pathway. There is an inflammation cascade which occurs. Generally the inflammation can result in an elimination of the irritant.

4. Chronic Inflammation: If the irritant is not eliminated or if it is ongoing such as ROS production due to dietary imbalances, then the result in a chronic inflammation. This is often the hallmark step leading to a cancer.

5. Dysplasia: The continual assault on the cells may result in blockage of genes or excess activation of growth factors. Also there may be significant methylation and resulting changes in promoters and expression.

6. Cancer: The final step may very well be the uncontrollable change to a malignancy.



14.2.2 Irritants: Reactive Oxygen Species, and Example

There are a multiplicity of irritants that initiate an immune system response. We use here a simple one that may be a likely potential chronic source, namely the reactive oxygen species or oxidized radicals. There are many others such as tobacco smoke, alcohol, drugs, and various types of foods. The example of the reactive oxygen species, ROS, is but one but it exemplary of the type seen. Specifically, ROS are prevalent in those with Type 2 Diabetes resulting from obesity. As such, and in view of the growing number of people subject to this self-inflicted disease, this specific example is worthy of some detailed attention as a prime example.

Let us begin with a discussion of ROS. As Cleveland and Kasten note:

Reactive oxygen species are potentially dangerous by-products of cellular metabolism that have direct effects on cell development, growth and survival, on ageing, and on the development of cancer. They are generated by all aerobic organisms, but their production is a double-edged sword. On the one hand, they seem to be needed for signal-transduction pathways that regulate cell growth and reduction– oxidation (redox) status. But on the other, excessive amounts of these metabolites can start lethal chain reactions, which oxidize and disable structures that are required for cellular integrity and survival1.

Many tumour cells seem to have increased rates of metabolism compared with normal cells, which would typically lead to increased numbers of reactive oxygen species. So one way of treating cancer might be to design drugs to target the enzymes that regulate the levels of reactive oxygen species….

Reactive oxygen species are generated during the production of ATP by aerobic metabolism in mitochondria. The leakage of electrons from mitochondria during the electron-transport steps of ATP production generates the reactive oxygen species superoxide (O2-) and hydroxyl (OH -) radicals. These species can lead to the production of hydrogen peroxide (H2O2), from which further hydroxyl radicals are generated in a reaction that either depends on, or is catalysed by, Fe 2+ ions.

Cells have evolved a series of antioxidant systems to handle these dangerous natural by-products. These defence systems include intracellular superoxide dismutases (SODs), which convert O2- into H2O 2; enzymes that inactivate H2O2 or hydroxyl radicals; and enzymes that trap free radicals or transition metals (such as Fe 2+) that are a reservoir for electrons.

It is well known that these radicals can have drastic inflammatory effects on cells. As Rubin and Strayer note:

Hydroxyl radicals (OH•) are formed by

(1) the radiolysis of water,

(2) the reaction of H_2O_2 with ferrous iron (Fe_2) (the Fenton reaction) and

(3) the reaction of O_2 with H_2O_2 (the Haber-Weiss reaction).

The hydroxyl radical is the most reactive molecule of ROS and there are several mechanisms by which it can damage macromolecules. Iron is often an active participant in oxidative damage to cells by virtue of the Fenton reaction. Many lines of experimental evidence now suggest that in a number of different cell types H_2O_2 stimulates iron uptake and so increases production of hydroxyl radicals.

Lipid peroxidation: The hydroxyl radical removes a hydrogen atom from the unsaturated fatty acids of membrane phospholipids, a process that forms a free lipid radical. The lipid radical, in turn, reacts with molecular oxygen and forms a lipid peroxide radical. This peroxide radical can, in turn, function as an initiator, removing another hydrogen atom from a second unsaturated fatty acid. A lipid peroxide and a new lipid radical result and a chain reaction is initiated. Lipid peroxides are unstable and break down into smaller molecules. The destruction of the unsaturated fatty acids of phospholipids results in a loss of membrane integrity.

Protein interactions: Hydroxyl radicals may also attack proteins. The sulfur-containing amino acids cysteine and methionine, as well as arginine, histidine and proline, are especially vulnerable to attack by OH•. As a result of oxidative damage, proteins undergo fragmentation, cross-linking, aggregation and eventually degradation.

DNA damage: DNA is an important target of the hydroxyl radical. A variety of structural alterations include strand breaks, modified bases and cross-links between strands. In most cases, the integrity of the genome can be reconstituted by the various DNA repair pathways. However, if oxidative damage to DNA is sufficiently extensive, the cell dies.

The DNA damage is a key factor. ROS when present especially in cells which undergo more rapid duplication where the DNA is exposed to the ROS are thus subject to assault and change. The self-protective repair mechanisms of DNA may not be adequate to make all corrections by eliminating defective DNA or properly repairing single or double stranded DNA breaks[70]. Again Grivennikov et al note:

It has been suggested that an inflammatory microenvironment can increase mutation rates, in addition to enhancing the proliferation of mutated cells. Activated inflammatory cells serve as sources of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that are capable of inducing DNA damage and genomic instability. However, it is not clear whether ROS and RNI produced and released by neutrophils or macrophages (mainly during acute inflammation) are sufficiently long lived to diffuse through the extracellular matrix, enter epithelial cells, cross their cytoplasm, enter the nucleus, and react with DNA packaged into chromatin.

Alternatively, inflammatory cells may use cytokines such as TNF-a to stimulate ROS accumulation in neighboring epithelial cells. It has therefore been debated whether immune-mediated mechanisms as opposed to dietary and environmental mutagens are the critical driving forces behind tumor initiation

The last sentence is a compelling statement. It simply states that the body's own immune system, driven by ROS, may be a source of many of the damages imparted upon DNA. Further research as reported by Gorlach et al notes:

Within the last twenty years the view on reactive oxygen species (ROS) has changed; they are no longer only considered to be harmful but also necessary for cellular communication and homeostasis in different organisms ranging from bacteria to mammals. In the latter, ROS were shown to modulate diverse physiological processes including the regulation of growth factor signaling, the hypoxic response, inflammation and the immune response.

During the last 60–100 years the life style, at least in the Western world, has changed enormously. This became obvious with an increase in caloric intake, decreased energy expenditure as well as the appearance of alcoholism and smoking; These changes were shown to contribute to generation of ROS which are, at least in part, associated with the occurrence of several chronic diseases like adiposity, atherosclerosis, type II diabetes, and cancer.…

[70] We have discussed the SS DNA and DS DNA repairs elsewhere. BRCA is the DSB repair protein and it is well known that in breast cancers and many others this lack of BRCA can lead to high malignancy. PARP is a SSB repair and it too, especially in prostate cancer, is a source of malignant transformation.

The research within the last twenty years on chemically reactive molecules containing oxygen, commonly called reactive oxygen species (ROS), has shown that these molecules are important for cellular communication and homeostasis in different organisms ranging from bacteria to mammals. Thereby, ROS were shown to modulate diverse physiological processes including the regulation of growth factor signaling, the hypoxic response, inflammation and the immune response in mammalian cells.

ROS are often simply called "free radicals" because their majority is characterized by at least one unpaired electron in their outer orbitals; however, peroxides like hydrogen peroxide may also give rise to the formation of oxygen radicals and are therefore also considered as ROS. Frequently the incomplete reduction of oxygen by one electron producing super oxide anion ($O2^{\cdot-}$) is the first step for the formation of most other ROS.

On the one hand we have managed to extend life span. On the other hand we have seen a dramatic increase in cancers due to longer life and more DNA repairs, but also as alleged the exposure to ROS in both environmental and dietary factors. As Alfadda et al note in their discussion of ROS in the context of the immune system:

Essentially, ROS are deeply involved in both arms of the immunological defense system, the innate and the acquired responses. Upon exposure to environmental pathogens, exaggerated ROS production as a part of the oxidative burst in activated phagocytes present in the local inflammatory milieu represents one of the first lines of defense mounted against the invading pathogens. Although rapid, this innate immunity is usually only partially effective, since certain fraction of pathogens might escape and proliferate, thereby producing a larger number of pathogens.

Acquired immunity will be initiated when pathogen-derived antigenic peptides that are the result of phagocytosis and digestion by activated phagocytes are presented to the T lymphocytes. As a result, the latter will proliferate and differentiate producing a large progeny of immunological effector cells that are capable of mounting an efficient and antigen-specific immune response. ROS are involved in the acquired immune response because excess ROS continue to be locally produced by the activated phagocytes and consequently enhance the intracellular signal transduction cascades within the T lymphocytes and thereby decrease their activation threshold

There is also the issue of which organs and/or cells are most susceptible to ROS compromise. Studies of the epithelial cells in the colon have been done extensively and putatively assigned high exposure. Yet the cells can easily defend themselves. Other cells may not be as well attuned to such defense.

14.2.3 Inflammation and Cancer

Inflammation has long been assumed to be a precursor and causative factor for cancers of all types. We first re-examine the nature of inflammation and then proceed to examine the relationship of inflammation to cancers.

We first address the issue of inflammation per se. As noted by Rubin and Styer:

Inflammation is a reaction, both systemic and local, of tissues and microcirculation to a pathogenic insult. It is characterized by elaboration of inflammatory mediators and movement of fluid and leukocytes from the blood into extravascular tissues. This response localizes and eliminates altered cells, foreign particles, microorganisms and antigens and paves the way for the return to normal structure and function. The clinical signs of inflammation, termed phlogosis by the Greek physician Galen, and inflammation in Latin, were described in classical times. In the first century AD , the Roman encyclopedist Aulus Celsus described the four cardinal signs of inflammation, namely, rubor (redness), calor (heat), tumor (swelling) and dolor (pain).

These features correspond to inflammatory events of vasodilation, edema and tissue damage. According to medieval concepts, inflammation represented an imbalance of various "humors," including blood, mucus and bile. Modern appreciation of the vascular basis of inflammation began in the 18th century with John Hunter, who noted dilation of blood vessels and appreciated that pus was accumulated material derived from the blood.

Rudolf Virchow first described inflammation as a reaction to prior tissue injury. To the four cardinal signs he added a fifth: functiolaesa (loss of function). Virchow's pupil Julius Cohnheim was the first to associate inflammation with emigration of leukocytes through the walls of the microvasculature. At the end of the19th century, the role of phagocytosis in inflammation was emphasized by the eminent Russian zoologist Eli Metchnikoff. Finally, the importance of chemical mediators was described in 1927 by Thomas Lewis, who showed that histamine and other substances increased vascular permeability and caused migration of leukocytes into extravascular spaces. More recent studies have elucidated the molecular and genetic bases of acute and chronic inflammation. …

They continue with the description of Chronic Inflammation:

When acute inflammation does not resolve or becomes disordered, chronic inflammation occurs. Inflammatory cells persist, stroma responds by becoming hyperplastic and tissue destruction and scarring lead to organ dysfunction. This process maybe localized, but more commonly it progresses to disabling dis-eases such as chronic lung disease, rheumatoid arthritis, asthma, ulcerative colitis, granulomatous diseases, autoimmune diseases and chronic dermatitis. Acute and chronic inflammation are ends of a dynamic continuum with overlap-ping morphologic features: (1) inflammation with continued recruitment of chronic inflammatory cells is followed by (2) tis-sue injury due to prolongation of the inflammatory response and (3) an often disordered attempt to restore tissue integrity. The events leading to amplified inflammatory responses resemble those of acute inflammation in a number of aspects:

1. *Specific triggers, microbial products or injury, initiate the response.*
2. *Chemical mediators: direct recruitment, activation and interaction of inflammatory cells. Activation of coagulation and complement cascades generates small peptides that function to prolong the inflammatory response.*
3. *Cytokines, specifically IL-6 and RANTES, regulate a switch in chemokines, such that mononuclear cells are directed to the site. Other cytokines (e.g., IFN-) then promote macrophage proliferation and activation.*

4. *Inflammatory cells: are recruited from the blood. Interac-tons between lymphocytes, macrophages, dendritic cells and fibroblasts generate antigen-specific responses.*
5. *Stromal cell activation and extracellular matrix remodel-in occur, both of which affect the cellular immune response.*

As Grivennikov et al in a recent review of Inflammation and Cancers note:

The presence of leukocytes within tumors, observed in the 19th century by Rudolf Virchow, provided the first indication of a possible link between inflammation and cancer. Yet, it is only during the last decade that clear evidence has been obtained that inflammation plays a critical role in tumorigenesis, and some of the underlying molecular mechanisms have been elucidated. A role for inflammation in tumorigenesis is now generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumors, including some in which a direct causal relationship with inflammation is not yet proven.

Only a minority of all cancers are caused by germline mutations, whereas the vast majority (90%) are linked to somatic mutations and environmental factors. Many environmental causes of cancer and risk factors are associated with some form of chronic inflammation. Up to 20% of cancers are linked to chronic infections, 30% can be attributed to tobacco smoking and inhaled pollutants (such as silica and asbestos), and 35% can be attributed to dietary factors (20% of cancer burden is linked to obesity).

It appears that the human cells can withstand a certain amount of antigenic assault but that a chronic assault is oftentimes the driver for carcinogenic change. They continue with their list of basic facts:

1. *Chronic inflammation increases cancer risk.*
2. *Subclinical, often undetectable inflammation may be as important in increasing cancer risk (for instance, obesity-induced inflammation).*
3. *Various types of immune and inflammatory cells are frequently present within tumors.*
4. *Immune cells affect malignant cells through production of cytokines, chemokines, growth factors, prostaglandins, and reactive oxygen and nitrogen species.*
5. *Inflammation impacts every single step of tumorigenesis, from initiation through tumor promotion, all the way to metastatic progression.*
6. *In developing tumors antitumorigenic and protumorigenic immune and inflammatory mechanisms coexist, but if the tumor is not rejected, the protumorigenic effect dominates.*
7. *Signaling pathways that mediate the protumorigenic effects of inflammation are often subject to a feed-forward loop (for example, activation of NF-kB in immune cells induces production of cytokines that activate NF-kB in cancer cells to induce chemokines that attract more inflammatory cells into the tumor).*
8. *Certain immune and inflammatory components may be dispensable during one stage of tumorigenesis but absolutely critical in another stage.*

Inflammation can be causative to cancers. The above authors (Grivennikov et al) note:

It has been suggested that an inflammatory microenvironment can increase mutation rates, in addition to enhancing the proliferation of mutated cells. Activated inflammatory cells serve as sources of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that are capable of inducing DNA damage and genomic instability. However, it is not clear whether ROS and RNI produced and released by neutrophils or macrophages (mainly during acute inflammation) are sufficiently long lived to diffuse through the extracellular matrix, enter epithelial cells, cross their cytoplasm, enter the nucleus, and react with DNA packaged into chromatin.

Alternatively, inflammatory cells may use cytokines such as TNF-a to stimulate ROS accumulation in neighboring epithelial cells. It has therefore been debated whether immune-mediated mechanisms as opposed to dietary and environmental mutagens are the critical driving forces behind tumor initiation. Nonetheless, p53 mutations, presumably caused by oxidative damage, were found in both cancer cells and in inflamed, but nondysplastic, epithelium in CAC, suggesting that chronic inflammation causes genomic changes. Chronic inflammation triggered by the colonic irritant dextran sodium sulfate (DSS) may induce DNA damage that gives rise to colonic adenomas. However, on its own DSS is a poor carcinogen.

However we also have many epigenetic factors as well. For example, methylation and acetylation play significant roles in activating and/or suppressing gene expressions. As to that the authors note:

Other findings implicate epigenetic mechanisms, including microRNA-based silencing and DNA methylation, in inactivation of tumor suppressors, such as INK4a and APC, and other changes that accompany tumor initiation. Recently, inflammation has been connected to epigenetic reprogramming by the JmjC-domain protein Jmjd3, which is encoded by an NF-kB target gene. In inflammation-associated intestinal cancer in Gpx1/2 knockout mice, inflammation induces DNA methyltransferase (DNMT)-dependent DNA methylation and silencing of a large cohort of Polycomb group target genes, some of which are also silenced by methylation in human colon cancer. However, it remains to be shown that any of these inflammation- induced epigenetic mechanisms actually make a critical contribution to tumor initiation, either in a suitable mouse model or through prospective analysis of human specimens.

We shall address this later as well as in an upcoming analysis of epigenetic factors.

Inflammation has become well known as a source of a multiplicity of pathological states. Hansson has described how it is a major source of coronary debilitation:

Recent research has shown that inflammation plays a key role in coronary artery disease (CAD) and other manifestations of atherosclerosis. Immune cells dominate early atherosclerotic lesions, their effector molecules accelerate progression of the lesions, and activation of inflammation can elicit acute coronary syndromes. This review highlights the role of inflammation in the pathogenesis of atherosclerotic CAD. It will recount the evidence that atherosclerosis, the main cause of CAD, is an inflammatory disease in which immune mechanisms interact with metabolic risk factors to initiate, propagate, and activate lesions in the arterial tree.

In addition the following is a list of the immune system cells and their positive and negative effects.

Cell	***Anti-Tumor***	***Tumor Promoting***
Macrophages, dendritic cells, myeloid-derived suppressor cells	Antigen presentation; production of cytokines (IL-12 and type I IFN)	Immunosuppression; production of cytokines, chemokines, proteases, growth factors, and angiogenic factors
Mast cells		Production of cytokines
B cells	Production of tumor-specific antibodies?	Production of cytokines and antibodies; activation of mast cells; immunosuppression
CD8+ T cells	Direct lysis of cancer cells; production of cytotoxic cytokines	Production of cytokines?
CD4+ Th2 cells		Education of macrophages; production of cytokines; B cell activation
CD4+ Th1 cells	Help to cytotoxic T lymphocytes (CTLs) in tumor rejection; production of cytokines (IFNg)	Production of cytokines
CD4+ Th17 cells	Activation of CTLs	Production of cytokines
CD4+ Treg cells	Suppression of inflammation (cytokines and other suppressive mechanisms)	Immunosuppression; production of cytokines
Natural killer cells	Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines	
Natural killer T cells	Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines	
Neutrophils	Direct cytotoxicity; regulation of CTL responses	Production of cytokines, proteases, and ROS

The adaptive immune system is now well studied and includes the complex interactions between B and T cells. We shall not provide any details herein and refer the reader elsewhere. (See Abbas et al).

The innate immune system and an old system which predates the more complex adaptive system. It is that system which attempts to promptly mitigate any attack on the organism.

Innate immunity, the first line of defense against infections, is phylogenetically the oldest part of the immune system. It coevolved with microbes to protect all multicellular organisms from infections. Some components of the mammalian innate immune system are remarkably similar to

components in plants and insects, suggesting that these appeared in common ancestors long ago in evolution.

For example, peptides that are toxic to bacteria and fungi, called defensins, are found in plants and mammals and have essentially the same tertiary structure in both life forms. A family of receptors that we will discuss in detail later in this chapter, called Toll-like receptors, recognize pathogenic microbes and activate antimicrobial defense mechanisms. Toll-like receptors are found in every life form in the evolutionary tree from insects up to mammals.

The major signal transduction pathway that Toll-like receptors engage to activate cells, called the NF-κB pathway in mammals, also shows remarkable evolutionary conservation. In fact, most of the mechanisms of innate immune defense that we will discuss in this chapter appeared very early in evolution, when the first multicellular organisms evolved, about 750 million years ago. An adaptive immune system, in contrast, is clearly recognizable only in vertebrates that appeared about 350 to 500 million years.

The following is a partial list of the Pattern Recognition Molecules, of the Innate Immune System[71]

PRR	***Location***	***Specific Examples***	***Ligands (PAMPs or DAMPs)***
TLRs	Plasma membrane and endosomal membranes of DCs, phagocytes, B cells, endothelial cells, and many other cell types	TLRs 1–9	Various microbial molecules including bacterial LPS and peptidoglycans; viral nucleic acids
NLRs	Cytosol of phagocytes, epithelial cells, and other cells	NOD1/2	Bacterial cell wall peptidoglycans
		NLRP family (inflammasomes)	Intracellular crystals (urate, silica); changes in cytosolic ATP and ion concentrations; lysosomal damage
RLRs	Cytosol of phagocytes and other cells	RIG-1, MDA-5	Viral RNA
CDSs	Cytosol of many cell types	AIM2; STING-associated CDSs	Bacterial and viral DNA
CLRs	Plasma membranes of phagocytes	Mannose receptor	Microbial surface carbohydrates with terminal mannose and fructose
		DC-sign	

[71] AIM2, Absent in melanoma; CDSs, cytosolic DNA sensors; CLRs, C-type lectin–like receptors; DAMP, damage-associated molecular pattern; DC, dendritic cells; MDA, melanoma differentiation-associated gene; NLRs, NOD-like receptors; NOD, nucleotide oligomerization domain; PAMP, pathogen-associated molecular pattern; RLRs, RIG-like receptors; SP-D, surfactant protein D; STING, stimulator of IFN genes; TLRs, toll-like receptors.

PRR	*Location*	*Specific Examples*	*Ligands (PAMPs or DAMPs)*
		Dectin-1, Dectin-2	Glucans present in fungal and bacterial cell walls
Scavenger receptors	Plasma membranes of phagocytes	CD36	Microbial diacylglycerides
***N*-Formyl met-leu-phe receptors**	Plasma membranes of phagocytes	FPR and FPRL1	Peptides containing *N*-formylmethionyl residues
Pentraxins	Plasma	C-reactive protein	Microbial phosphorylcholine and phosphatidylethanolamine
Collectins	Plasma	Mannose-binding lectin	Carbohydrates with terminal mannose and fructose
	Alveoli	Surfactant proteins SP-A and SP-D	Various microbial structures
Ficolins	Plasma	Ficolin	*N*-acetylglucosamine and lipoteichoic acid components of the cell walls of gram-positive bacteria
Complement	Plasma	Various complement proteins	Microbial surfaces

The innate system is composed of two general categories; cellular and humoral. Specifically:

Cellular: This is the use of the NK, macrophage, mast, and dendritic cells. Namely, the Cellular Innate system is composed of those cells which can be activated by the presence of some readily recognized antigen.

Humoral: The humoral arm of the innate system is that portion where various molecules, proteins, flowing in the blood stream and outside the blood stream can seek out and respond by themselves to an antigen. A classic example is the three complement cascades; classic, alternative and lectin. These molecules by themselves can identify, attack, and destroy invaders. This part of the immune system is often overlooked as a tool in fighting malignancies. However, it is a tool used by the body to respond to inflammatory events, and in turn its over expression can often initiate cancers.

The humoral system is composed of four general elements. From Abbas et al:

Several different kinds of molecules that recognize microbes and promote innate responses exist in soluble form in the blood and extracellular fluids. These molecules provide early defense against pathogens that enter the circulation or are present outside host cells at some stage of their life cycle.

The soluble effector molecules function in two major ways:

1. By binding to microbes, they act as opsonins and enhance the ability of macrophages and neutrophils to phagocytose the microbes. This is because the phagocytic cells express membrane receptors specific for the opsonins, and these receptors can efficiently mediate the internalization of the complex of opsonin and bound microbe and subsequent destruction of the ingested microbe.

2. After binding to microbes, soluble mediators of innate immunity promote inflammatory responses that bring more phagocytes to sites of infections, and they may also directly kill microbes.

3. The soluble effector molecules are sometimes called the humoral branch of innate immunity, analogous to the humoral branch of adaptive immunity mediated by antibodies. The major components of the humoral innate immune system are the complement system, collectins, pentraxins, and ficolins, …

They are:

1. Complement: This is the complex cascade of molecules which when activated attack and drill holes in the presenting pathogen.

2. Contact Cascade:

3. Naturally Occurring Antibodies

4. Pentraxins: These are proteins which can identify and initiate an attack on an invader. They may precede a Complement attack.

14.2.4 Complement

The complement element is a chain or reactions resulting in the MAC, a set of proteins which drill a hole in a cell and result in its demise.

Now from Shishido et al:

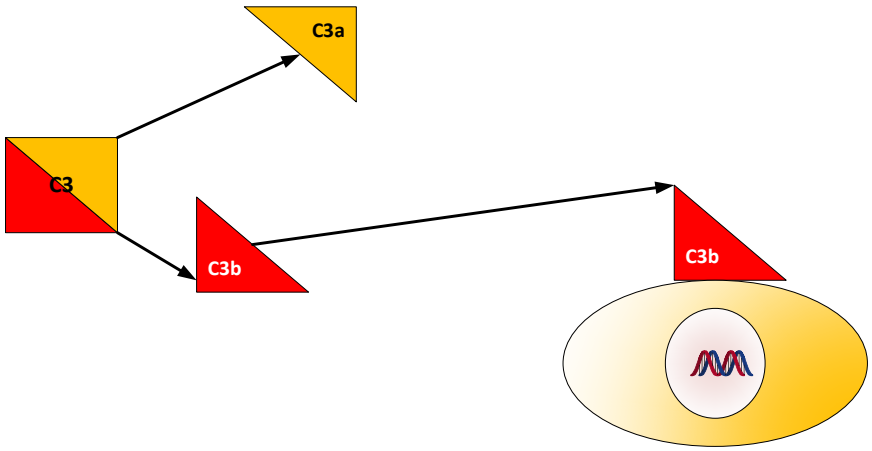
Complement activation occurs by one of three initiation pathways, the classical, alternative, or mannose binding lectin (MBL) pathway. Each pathway contains a C3 convertase that cleaves C3 producing C3b and subsequently a C5 convertase. Cleavage of C5 by the C5 convertase results

in C5b deposition and initiates the common terminal pathway. The terminal pathway forms the membrane attack complex (MAC), a pore in the cellular membrane, and lysis of the host or pathogenic cell. The action of the C3 and C5 convertases also produces potent anaphylatoxins, C3a and C5. Although not specifically part of the humoral immune response, complement receptor 3 (CR3) found on neutrophils and macrophages enhances the innate immune response by recognizing C3b opsonized pathogens.

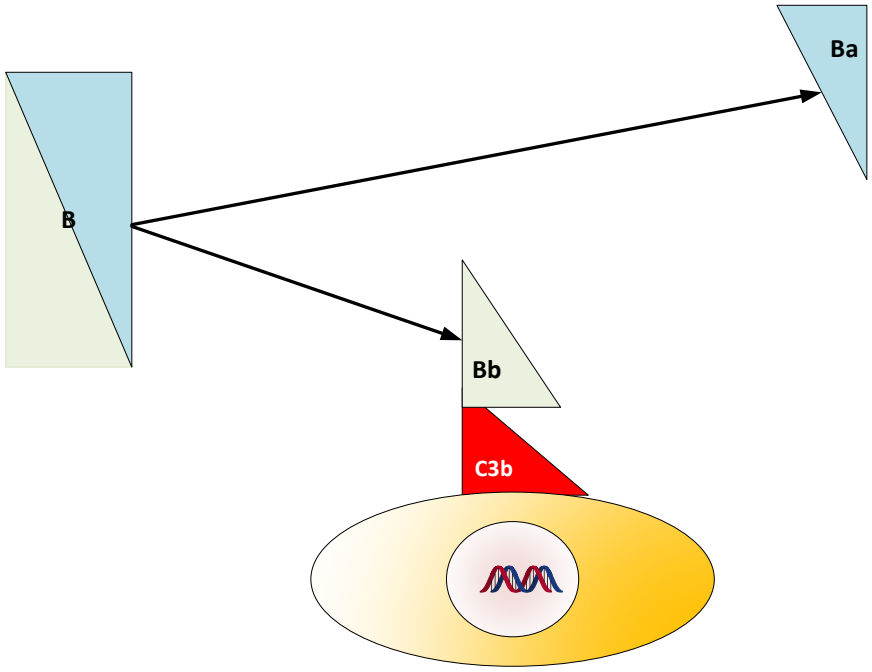
Recent evidence indicates that complement plays a significant role in directing the adaptive immune response as well as in tissue regeneration. Specifically, as part of the B cell receptor complex, CR2 recognition of cleavage products iC3b, C3dg, and C3d significantly increases Ab production. Thus, maintaining homeostasis requires tight regulation of the cascade. Regulation of this potentially damaging cascade occurs at multiple levels with soluble and membrane bound inhibitors including C1 inhibitor (C1INH), CD55, CD59, CD46, Factor H and related proteins.

TLR	Immune Cell Expression	PAMPs	DAMPs
TLR1+ TLR2	Cell surface	Triacylated lipoproteins (Pam3CSK4)	(TLR2 DAMPs listed below)
	Mo, MΦ, DC, B	Peptidoglycans, Lipopolysaccharides	
TLR2+ TLR6	Cell surface	Diacylated lipoproteins	Heat Shock Proteins
	Mo, MΦ, MC, B	(FSL-1)	(HSP 60, 70, Gp96)
			High mobility group proteins (HMGB1)
			Proteoglycans
			(Versican, Hyaluronic Acid fragments)
TLR3	Endosomes	dsRNA (poly (I:C))	mRNA
	B, T, NK, DC	tRNA, siRNA	tRNA
TLR4	Cell surface/	Lipopolysaccharides (LPS)	Heat Shock Proteins
	endosomes	Paclitaxel	(HSP22, 60, 70,72, Gp96)
	Mo, MΦ, DC, MC,		High mobility group proteins (HMGB1)
	IE		Proteoglycans
			(Versican, Heparin sulfate,
			Hyaluronic Acid fragments)
			Fibronectin, Tenascin-C
TLR5	Cell surface	Flagellin	
	Mo, MΦ, DC, IE		
TLR7	Endosomes	ssRNA	ssRNA
	Mo, MΦ, DC. B	Imidazoquinolines (R848)	
		Guanosine analogs (Loxoribine)	
TLR8	Endosomes	ssRNA,	ssRNA
	Mo, MΦ, DC, MC	Imidazoquinolines (R848)	
TLR9	Endosomes	CpG DNA	Chromatin IgG complex
	Mo, MΦ, DC, B,T	CpG ODNs	
TLR10	Endosomes	profilin-like proteins	
	Mo, MΦ, DC		

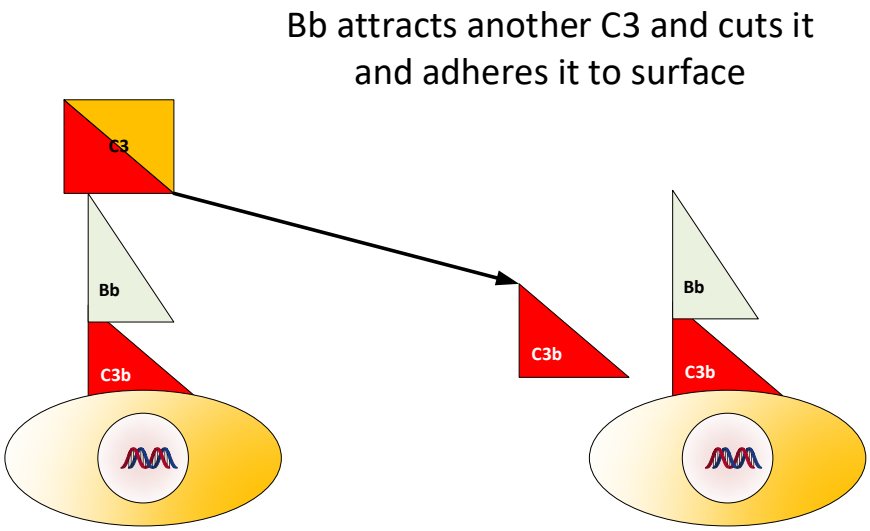
The following graphics depict the activation and actions of the complement system. First we have circulating C3 which can be broken into two active parts.C3b can attach to invading cells and this attachment starts the overall complement cascade. The alternative pathway is exemplary of this. One may ask how this could apply to invading cancer cells and we shall discuss this later.



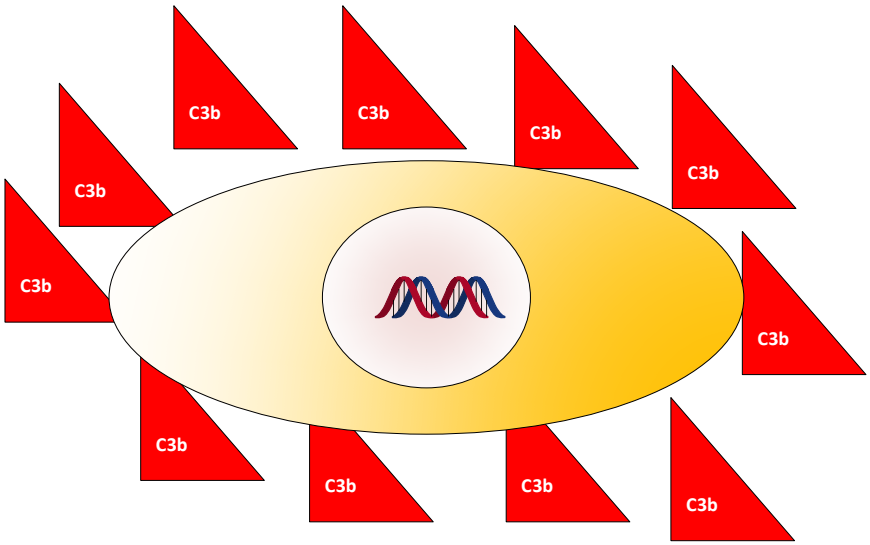
After the C3b is attached a B molecule binds to the c3B which itself is attached to the invader.



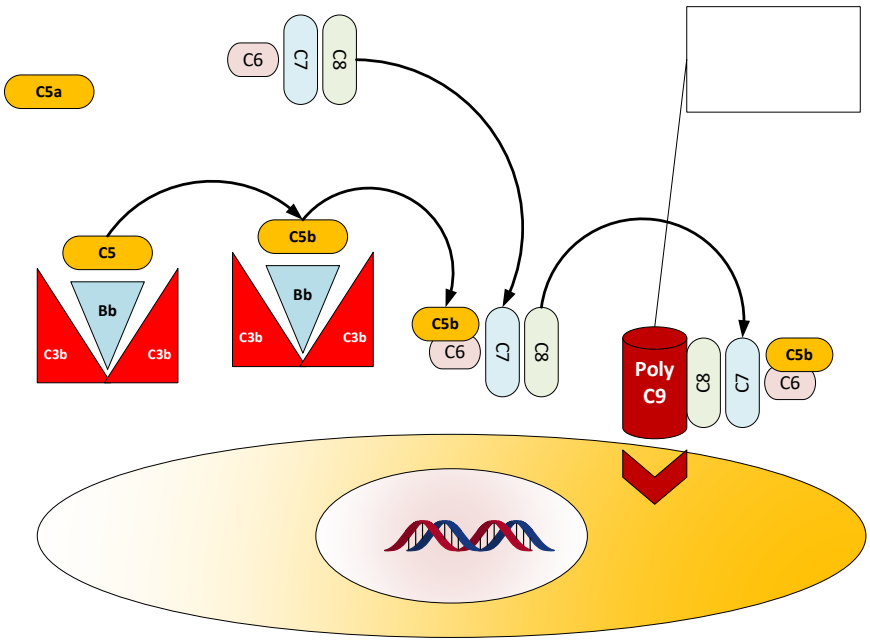
This initiates the C3b cascade wherein the C3 breaks apart and more and more C3b attach and cover the invader.



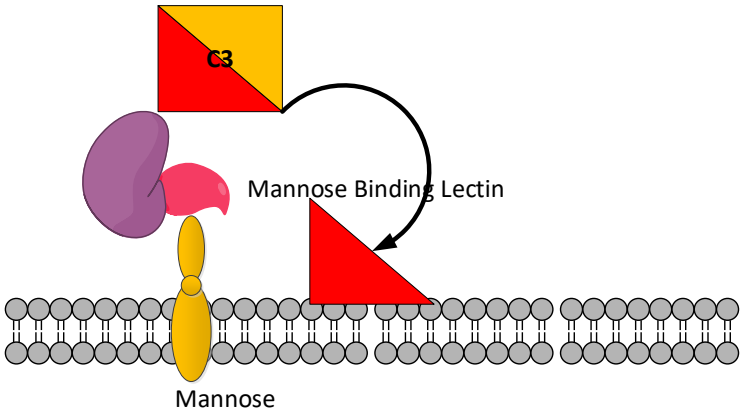
Finally the invader is totally covered with C3b.



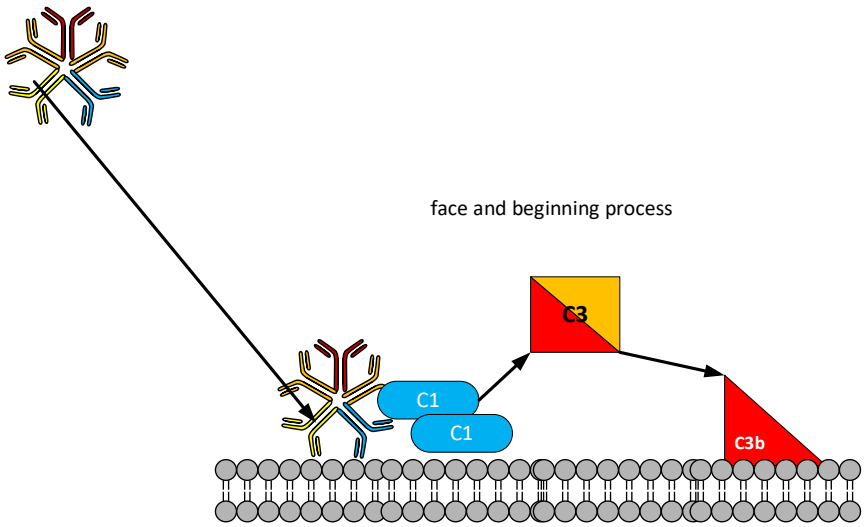
Finally the MAC or membrane attack complex is formed. This is an amalgam of a set of complement proteins which manages to attach to the invaders surface and drill holes through it that result in the death of the invader. It is a very powerful tool that can be used to seek out and destroy certain cells which have been targeted via the complement pathway.



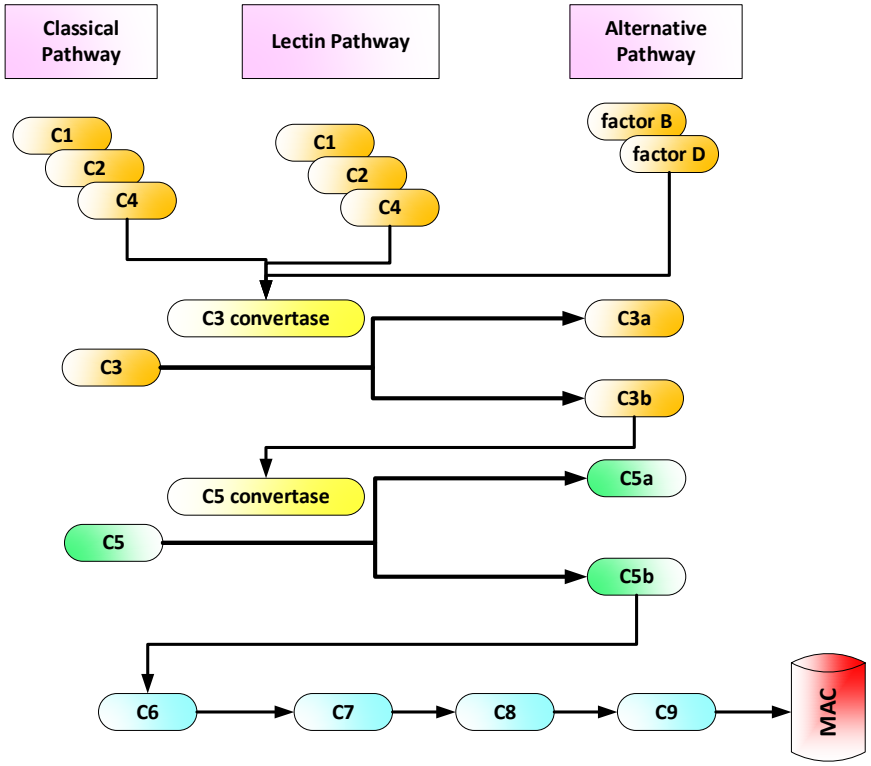
There are other ways in which complement works. The mannose pathway is shown below.



The third way, the classical, is shown below activated by an antibody.



We summarize these pathways below. Our main focus will be on the alternative. Mannose and classic require other surface molecules.



14.2.5 Contact cascade

Now from Shishido et al we have what the authors call the Contact Cascade:

The plasma also contains components of a second proteolytic cascade, the contact system. Factor XII (FXII; Hageman factor) of the contact system is proteolytically cleaved to FXIIa by

negatively charged surfaces of damaged cells. FXIIa initiates the coagulation cascade leading to clot formation and cleaves prekallikrein to kallikrein for subsequent release of bradykinin.

Through an endothelial G-coupled receptor (bradykinin receptor 1; BKR1), bradykinin induces vasodilation, neutrophil chemotaxis and vascular permeability. Furthermore, the bradykinin degradation product, des-arg9-bradykinin regulates the adaptive response and alters the blood-brain barrier through a second receptor, bradykinin receptor 2 (BKR2).

Importantly, both FXIIa and kallikrein activate the complement cascade independent of known complement initiators. Several components of the activated contact system including, FXa, FXIa and plasmin, cleave C5 and C3 producing C5a and C3a. The complement inhibitor, C1INH, also inhibits FXIIa indicating multiple interactions between the two pathways. These data suggest crosstalk between two cascades of humoral innate immune response.

14.2.6 Naturally occurring antibodies

Now from Shishido et al:

Produced primarily by B1 B lymphocytes, NAb are germline encoded Ab with restricted epitope specificities and are produced in the absence of external antigen stimulations. NAb are usually of the IgM isotype but may include IgG and IgA isotypes as well.

Natural IgM Abs mediate clearance of cellular debris, aging or apoptotic cells by oponization and recruitment of complement components. As part of the innate immune response, NAb recognize a wide range of pathogens, albeit with low affinity and modulate the adaptive immune response by interacting with B, T and dendritic cells [10]. Finally, NAb are potent initiators of the complement cascade suggesting additional interactions between components of the innate humoral response.

14.2.7 Pentraxins

The fourth and a significant part of the humoral innate system is the pentraxin. We shall focus on this element and discuss its usefulness as both a prognostic element and target for therapy. Now again from Shishido et al:

As a family of evolutionarily conserved multimeric pattern recognition proteins, pentraxins are acute phase proteins which are rapidly synthesized and serve as markers of infection, inflammation, and tissue damage. Each pentraxin contains a common domain in the C terminus. The presence or absence of additional domains divides the family into long or short pentraxins, respectively. The short pentraxins include C-reactive protein (CRP) and serum amyloid P protein (SAP), both of which are produced by the liver.

Produced by a multitude of cell types, pentraxin 3 (PTX3) is the primary long pentraxin active in humoral innate immunity. Similar to NAb, pentraxins recognize and bind multiple pathogens as well as intrinsic ligands, including apoptotic cells and extracellular matrix components. Macrophages and other innate immune cells recognize pentraxins, CRP, SAP and PTX3, via Fcy

receptors. Binding of pentraxins to a target facilitates clearance of pathogens and cell debris by complement activation indicating additional interactions between components of the innate immune response. Overall, pentraxins are multifunctional and nonredundant components of the humoral innate immune response.

Therefore, pentraxins play a critical role in human disease by interacting with multiple components of the humoral response.

We shall detail the pentraxins later in this report.

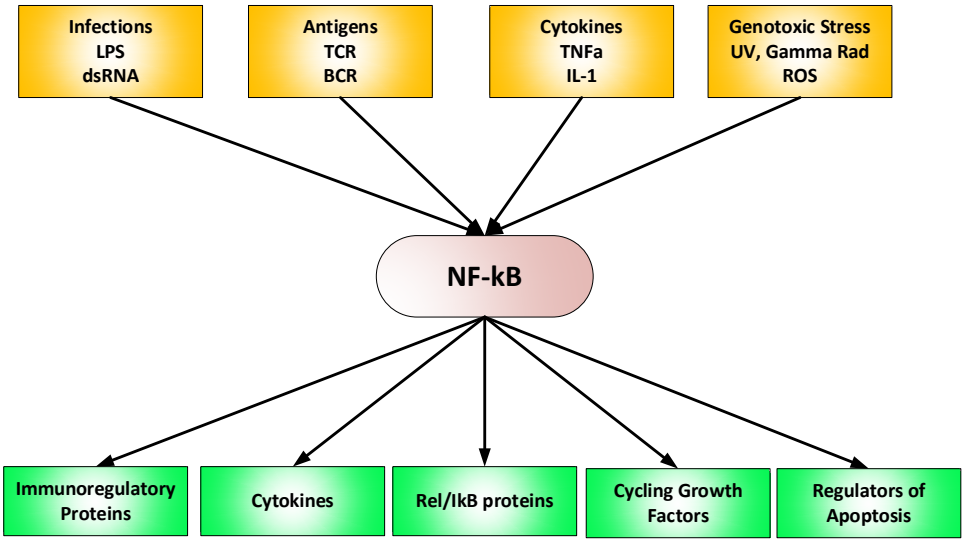
These four humoral innate elements have received some attention but they may be worth a considerable amount more as regards to the link between inflammation and cancer.

14.2.8 NF-kB and Its Implications

The NF-kB dimer is a powerful transcription factor that plays a role in the function of the immune system and in dealing with inflammation. It also has a significant role in cancer development. We briefly summarize this significant factor and highlight the key elements that relate to the conjunction between inflammation and cancer.

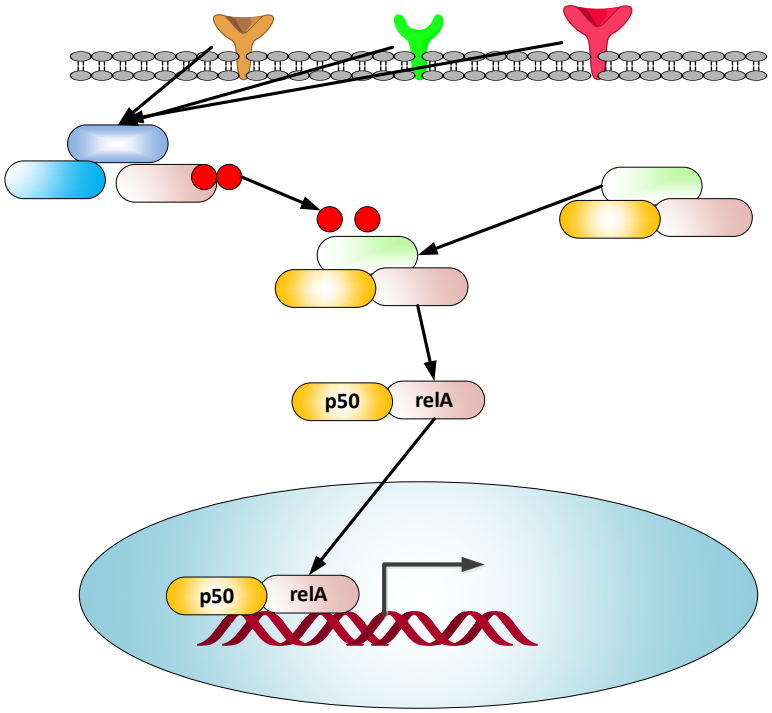
From Gorlach et al we have:

The activation of NF-κB is closely linked with ROS generation during inflammation and obesity. ROS were found to mediate inhibitor of NF-κBα (IκBα) kinase (IKKα and IKKβ) phosphorylation and release of free NF-κB dimers. Tumor necrosis factor α (TNFα), a bona fide NF-κB activator, was shown to mediate a redox- dependent activation of protein kinase A which subsequently phosphorylated Ser276 on RelA (v-rel avian reticuloendotheliosis viral oncogene homolog A). By contrast, the NF-κB member p50 was found to have reduced DNA binding activity when oxidized at Cys62.

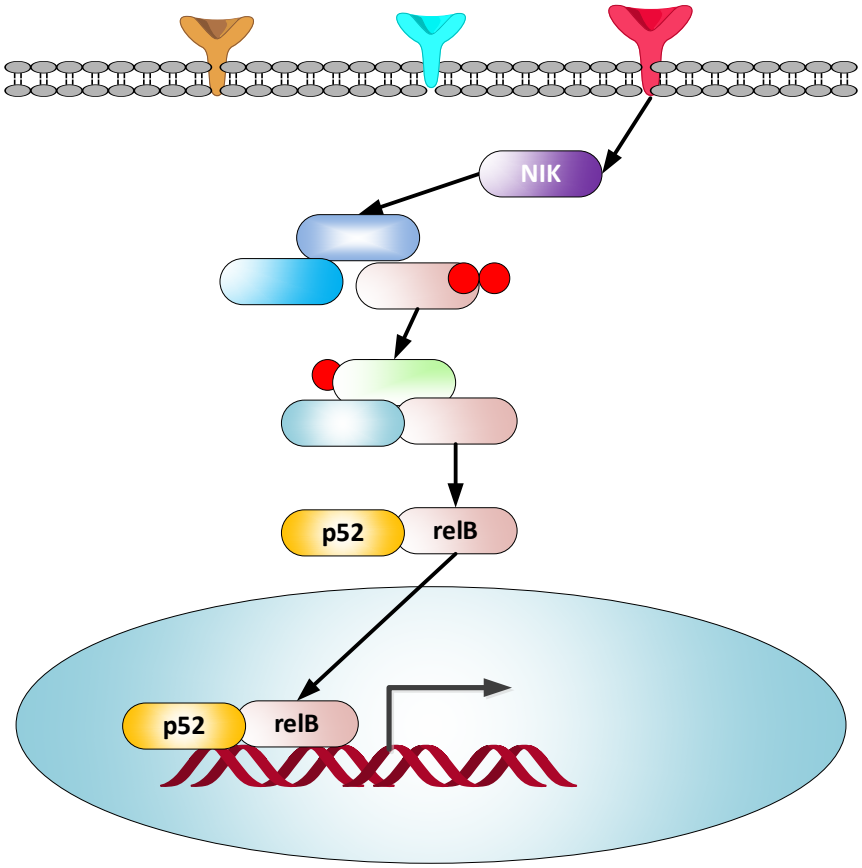


Note that NF-kB can be activated by the very things that are part of the inflammatory response. In turn, NF-kB as a promoter can then release more of this drivers, increase growth factor expression, and stop normal apoptosis. NF-kB is one of the most significant intracellular drivers that connects inflammation, the immune system, and unregulated growth.

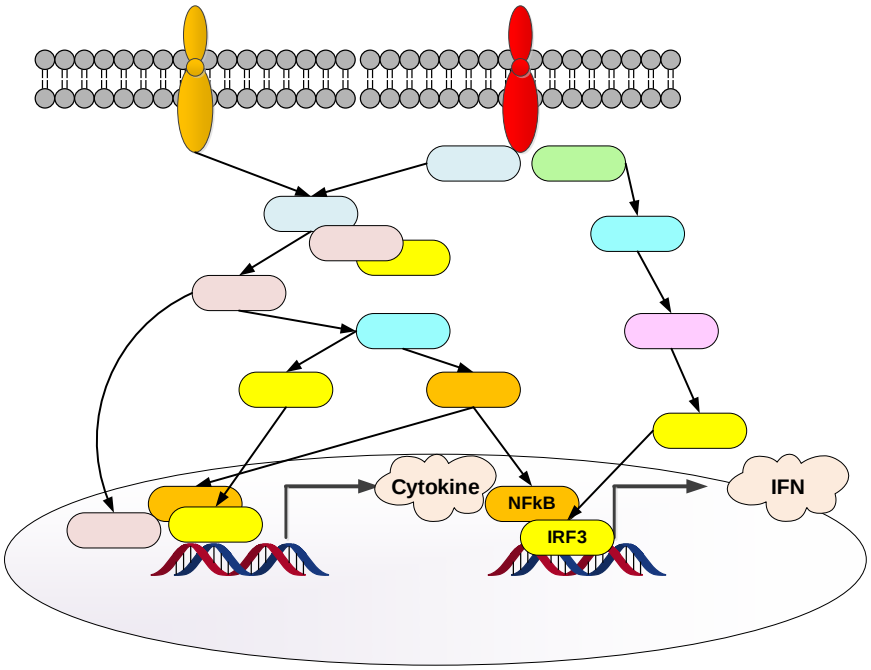
Now as we noted NF-kB is a dimer, namely a combination of two proteins which in turn, when activated, result in a molecule which in a very effective promoter. We show this below with a set of examples:



Here in the above is one of the dimer expressed, namely a relA along with a p50. Below we show the relB and the p52 expression.



The integrated combination is shown below.



To understand NF-kB we need to see how it functions as a powerful promoter. From NCBI we have the following description[72]:

NF-kappa-B is a ubiquitous transcription factor involved in several biological processes. It is held in the cytoplasm in an inactive state by specific inhibitors. Upon degradation of the inhibitor, NF-kappa-B moves to the nucleus and activates transcription of specific genes. NF-kappa-B is composed of NFKB1 or NFKB2 bound to either REL, RELA, or RELB. The most abundant form of NF-kappa-B is NFKB1 complexed with the product of this gene, RELA. Four transcript variants encoding different isoforms have been found for this gene.

From Nature we have the following description[73]:

The canonical pathway is induced by tumour necrosis factor-alpha (TNFalpha), interleukin-1 (IL-1) and many other stimuli, and is dependent on activation of IKKbeta. This activation results in the phosphorylation (P) of IkappaBalpha at Ser32 and Ser36, leading to its ubiquitylation (Ub) and subsequent degradation by the 26S proteasome. Release of the NF-kappaB complex allows it to relocate to the nucleus. Under some circumstances, the NF-kappaB–IkappaBalpha complex shuttles between the cytoplasm and the nucleus (not shown).

IKK-dependent activation of NF-kappaB can occur following genotoxic stress. Here, NF-kappaB essential modifier (NEMO) localizes to the nucleus, where it is sumoylated and then ubiquitylated, in a process that is dependent on the ataxia telangiectasia mutated (ATM) checkpoint kinase. NEMO relocates back to the cytoplasm together with ATM, where activation of IKK-beta occurs. IKK-independent atypical pathways of NF-kappaB activation have also been described, which include casein kinase-II (CK2) and tyrosine-kinase-dependent pathways.

The non-canonical pathway results in the activation of IKK alpha by the NF-kappa B-inducing kinase (NIK), followed by phosphorylation of the p100 NF-kappa B subunit by IKK alpha. This results in proteasome-dependent processing of p100 to p52, which can lead to the activation of p52–Rel B heterodimers that target distinct kappa B elements. Phosphorylation of NF-kappa B subunits by nuclear kinases, and modification of these subunits by acetylases and phosphatases, can result in transcriptional activation and repression as well as promoter-specific effects.

Moreover, cooperative interactions with heterologous transcription factors can target NF-kappa B complexes to specific promoters, resulting in the selective activation of gene expression following cellular exposure to distinct stimuli.

As Merle et al discuss when examine the Complement system they state:

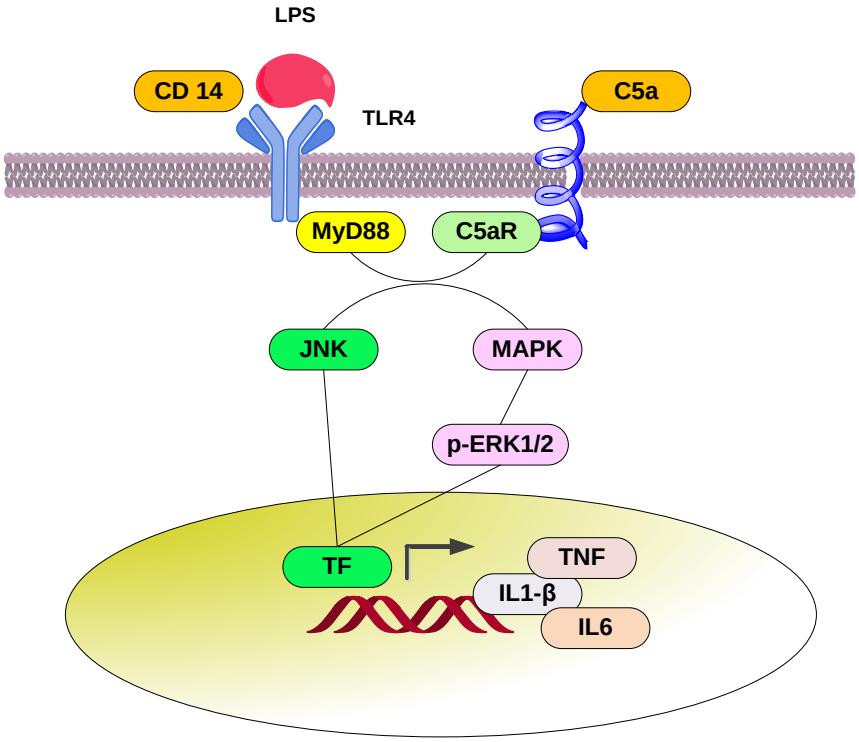
C3a and C5a are able to induce potent inflammatory pathways via their receptors C3aR and C5aR. The implication of intermediates such as NF-kB, MAPK, and c-Jun N-terminal kinase

[72] https://www.ncbi.nlm.nih.gov/gene/5970

[73] https://www.nature.com/nrm/journal/v8/n1/box/nrm2083_BX1.html

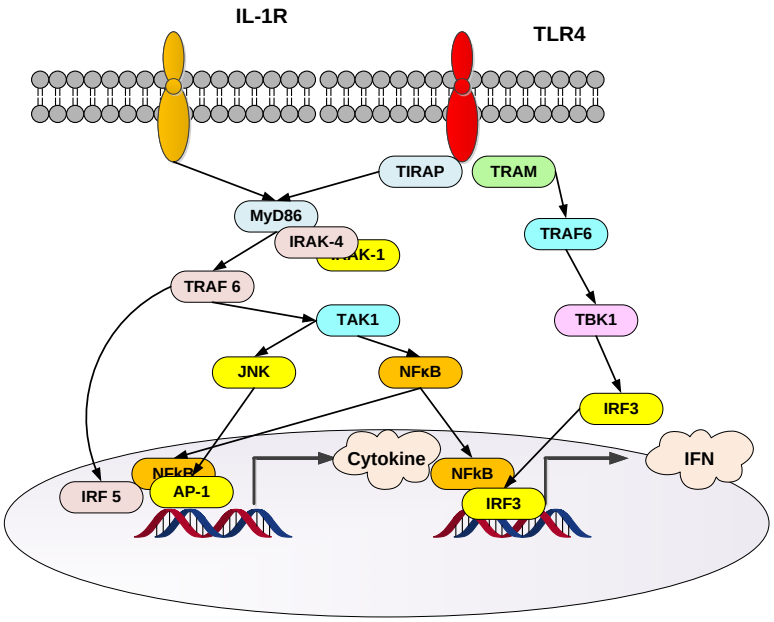
(JNK) in their transduction pathways suggests a potential crosstalk with other pathways, such as those of TLRs. Indeed, complement is involved in TLR-induced inflammation.

They show in the following Figure how this does function:



C5a/C5aR signalingpathwaycancooperatewithTLR-4activationby LPS on macrophages. Intermediate signaling pathways JNK and MAPK are activated and thus lead to proinflammatory effect by TNF-α, IL6, and IL1-β synthesis. On dendritic cells (DCs),TLR-4 and C5aR cooperate in different manner between mice and human. In vivo experiments have demonstrated an implication in Th1cells expansion, whereas in human, an anti-inflammatory role of TLR-4/C5aR collaboration has been described by an antagonized effect on IL-12 and IL-23 synthesis by DC.

Thus, when examining the effects of the complement proteins one must also examine the interactions with other receptors. Further details on this interaction are shown below. Here we show the Toll like receptors, TLR as initiations. These are powerful initiators in the innate response.



As Amiri and Richmond state:

Nuclear Factor-kappa B (NF-κB) is an inducible transcription factor that regulates the expression of many genes involved in the immune response. Recently, NF-κB activity has been shown to be upregulated in many cancers, including melanoma. Data indicate that the enhanced activation of NF- κB may be due to deregulations in upstream signaling pathways such as Ras/Raf, PI3K/Akt, and NIK. Multiple studies have shown that NF-κB is involved in the regulation of apoptosis, angiogenesis, and tumor cell invasion, all of which indicate the important role of NF-κB in tumorigenesis. Thus, understanding the molecular mechanism of melanoma progression will aid in designing new therapeutic approaches for melanoma.

They continue:

Constitutive activation of NF-κB is an emerging hallmark of various types of tumors including breast, colon, pancreatic, ovarian, and melanoma. In the healthy human, NF-κB regulates the expression of genes involved in normal immunologic reactions (e.g. generation of immunoregulatory molecules such as antibody light chains) in response to proinflammatory cytokines and by-products of microbial and viral infections. NF-κB also modulates the expression of factors responsible for growth as well as apoptosis. However, increased activation of NF-κB results in enhanced expression of proinflammatory mediators, leading to acute inflammatory injury to lungs and other organs, and development of multiple organ dysfunctions as well as cancer.

They then summarize NF-kB's role as:

***3.1. Apoptosis resistance and cell proliferation**: In processes such as tumor initiation and promotion where prolonged survival of cells is a crucial event, NF-κB plays an important role as a mediator of inhibition of apoptosis. In melanoma, NF-κB has been shown to activate*

expression of anti-apoptotic proteins such as tumor necrosis factor receptor-associated factor 1 (TRAF1), TRAF2, and the inhibitor-of apoptosis (IAP) proteins c-IAP1, c-IAP2, and melanoma inhibitor of apoptosis (ML-IAP), survivin as well as Bcl-2 like proteins…

***3.2. Invasion and metastasis**: In invasion and metastasis of melanoma, NF-κB may regulate the production of prostaglandins via cyclooxygenase-2 (COX-2), which has been shown to be overexpressed in melanoma [44,45]. It was shown that COX-2 is expressed in the majority of primary malignant melanoma, as well as in five human malignant melanoma cell lines….*

However as Liu et al (2006) state:

Malignant melanoma is the most lethal skin cancer, whose ability to rapidly metastasize often prevents surgical cure.

Furthermore, the systemic treatment of melanoma is largely ineffective due to the intrinsic resistance of melanoma cells to numerous anticancer agents. Increased survival of melanoma cells is primarily attributed to the constitutive activation of the transcription factor nuclear factor kB (NF-kB), which regulates the expression of many anti-apoptotic, pro-proliferative and pro-metastatic genes.

Canonical activation of the NF-kB pathway occurs when NF-kB switches its localization from the cytoplasm, where it is maintained inactive by assembly with the inhibitor IkB protein, to the nucleus, where NF-kB regulates gene expression. NF-kB activation relies upon the phosphorylation dependent ubiquitination and degradation of IkB mediated by the IkB kinase (IKK) complex and b-Trcp E3 ubiquitin ligases.

Consequently, both IKK activity and the levels of b-Trcp regulate the extent of IkB degradation and hence NF-kB activation. The genetic basis that underlies the elevated NF-kB activity in malignant melanoma largely remains elusive.

Constitutively active IKK has been demonstrated to sustain NF-kB activation in human melanoma cells, resulting in induction of the chemokine CXCL1. CXCL1, in turn, is capable of activating IKK and NF-kB and promoting cell survival and tumorigenesis …. However, the original genetic alterations that initiate this feed-forward mechanism in melanoma remain unclear.

One of the major oncogenic events described in the genesis of malignant melanoma is constitutive activation of the Ras-regulated RAF-MEK-ERK mitogen-activated protein kinase (MAPK) pathway. This is achieved most frequently by activating mutations in either BRAF (e.g. V600E substitution) or, less frequently, in N-RAS … Recent evidence indicates that oncogenic BRAF activity is essential for human melanoma cell growth and survival …

However, despite prior reports that RAF can activate NF-kB …, the mechanism(s) by which $BRAF_{V600E}$ ($BRAF_{VE}$) may elicit NF-kB signaling in melanoma cells have not yet been elucidated. Activation of the canonical NF-kB pathway depends on both IKK activity, which has been shown to be elevated in human melanomas….

Liu et al conclusion is speculative but telling:

Taken together, these data support a model in which mutational activation of BRAF in human melanomas contributes to constitutive induction of NF-κB activity and to increased survival of melanoma cells.

Again we have the issue of speculation as to where and why the mutations occur. Here they speculate about the BRAF mutation resulting in the antiapoptotic control with NF-κB.

14.2.9 Pentraxin 3

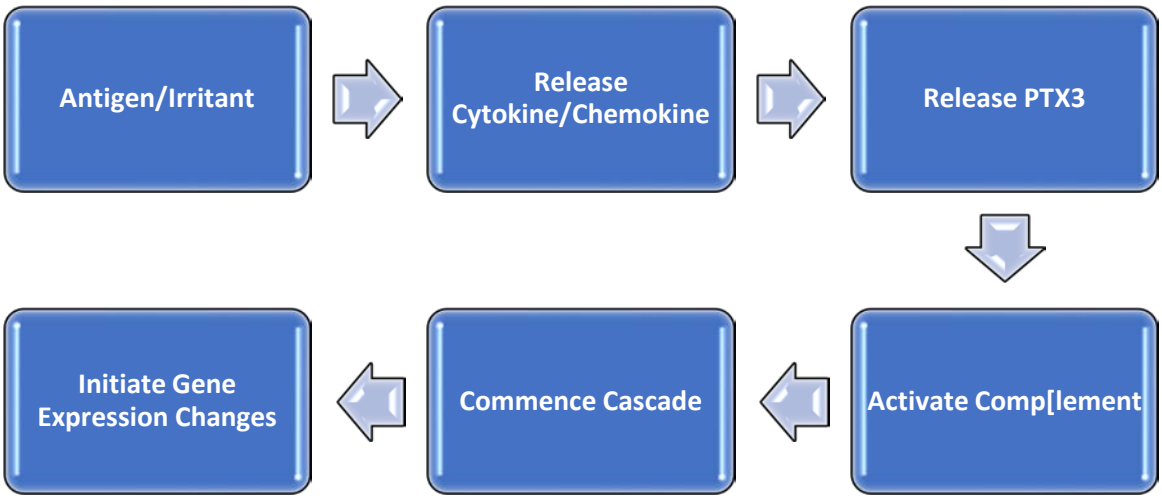
Pentraxin is one of the four generic elements of the humoral innate system. Generally the emphasis is on complement but Pentraxin presents a powerful element not only to monitor progress of inflammation but also to assess the impact and finally as a putative therapeutic target for cancer management. We examine PTX3 as an example of the immune system itself playing a part in the context of inflammation response and the resulting changes that impact malignant behavior.

As NCBI notes[74]:

This gene encodes a member of the pentraxin protein family. The expression of this protein is induced by inflammatory cytokines in response to inflammatory stimuli in several mesenchymal and epithelial cell types, particularly endothelial cells and mononuclear phagocytes. The protein promotes fibrocyte differentiation and is involved in regulating inflammation and complement activation. It also plays a role in angiogenesis and tissue remodeling. The protein serves as a biomarker for several inflammatory conditions.

As noted above the PTX3 produced can be a powerful marker. This is especially the case with a multiplicity of inflammatory processes.

[74] https://www.ncbi.nlm.nih.gov/gene/5806



We now examine the impact that PTX has. From Abbas et al we have:

PTX3 is

(i) produced by several cell types, including DCs, macrophages, and endothelial cells,

(ii) in response to TLR ligands and inflammatory cytokines, such as TNF, and may be considered an acute-phase reactant.

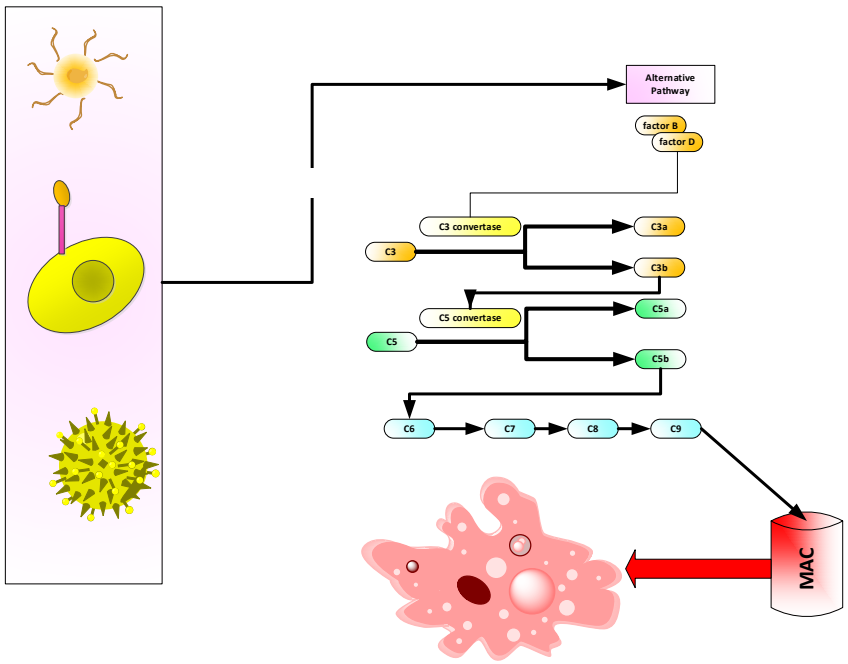
(iii) PTX3 is also stored in neutrophil granules and released as neutrophils die.

(iv) PTX3 recognizes various molecules on fungi, certain bacteria, and viruses, as well as apoptotic cells, and

(v) activates the classical complement pathway.

Studies with knockout mice reveal that PTX3 provides protection against these microbes, including the fungus….

We demonstrate this process below:



The use of PTX3 is the activation of the classical complement pathway. It should be recalled, however, that complement does require an Ab presence of some sort.

Now let us consider PTX3 from the perspective of Bonavita et al:

Inflammation is an essential component of the tumor microenvironment that sustains tumor development and growth. The role in cancer-related inflammation of innate immunity cells recruited in the tumor has been clarified in preclinical models. In contrast, the role of the humoral arm of the innate immune system, which includes biochemically heterogeneous molecules such as Complement components, collectins, ficolins and pentraxins, is still under investigated.

The long pentraxin PTX3 represents a functional paradigm of humoral innate immunity. By interacting with selected microbial moieties and playing opsonic activity via Fcγ receptors, and activating and regulating the Complement cascade, PTX3 acts as a functional ancestor of antibodies. PTX3 plays non-redundant roles in resistance against selected microbial pathogens and in regulating inflammatory and tissue repair responses. PTX3 is highly conserved in evolution and genetic evidence is consistent with a role of PTX3 in antimicrobial resistance in humans. ….

We also showed that PTX3-deficiency was associated to increased DNA damage, as demonstrated by increased Trp53 mutations, oxidative DNA damage and expression of DNA damage (DDR) markers, in line with the hypothesis that cancer-associate inflammation contributes to genetic events that cause cancer and to the genetic instability of tumors.

We finally showed that PTX3 promoter and regulatory regions were highly methylated in selected human mesenchymal and epithelial tumors, in contrast to the normal counterpart. In particular, in colorectal cancer, PTX3 epigenetic modifications occurred early in progression

already at the level of adenomas. PTX3 methylation was responsible of silencing of PTX3 protein expression. Indeed, treatment of colorectal cancer cells with a methylation inhibitor (5-Aza-2'- deoxycytidine) was sufficient to restore the histone modifications associated to transcriptional activation and the interaction of transcription factors responsible of PTX3 expression (e.g. NF-kB, c-Jun, c-Fos) with their binding sites in the PTX3 promoter region, and rescued PTX3 protein expression in response to an inflammatory stimulus.

The following relates to the production of PTX3[75]:

PTX3 is rapidly induced by several stimuli in different cell types. Peripheral blood leukocytes and myeloid dendritic cells (DCs) release PTX3 in response to proinflammatory cytokines (IL-1 and TNF-α) and agonists of TLR or following stimulation with microbial components, including LPS, lipoarabinomannan, and Outer membrane proteins (Omp).

PTX3 production is also stimulated by the anti-inflammatory cytokine IL-10 and by high-density lipoproteins (HDLs). Polymorphonuclear cells (PMNs) store PTX3 in lactoferrin-positive granules. Following microbial recognition by cellular pattern-recognition receptors, PTX3 is promptly released from PMN granules and localizes in neutrophil extracellular traps, where it likely contributes to the generation of an antimicrobial microenvironment essential to trap and kill microorganisms. Other cell types can produce PTX3 in response to appropriate proinflammatory stimulation, such as vascular ECs; smooth muscle cells (SMCs); fibroblasts; adipocytes; chondrocytes; stromal, mesangial, and epithelial cells; and cells of the granulosa.

Different signaling pathways can affect PTX3 production, depending on the cell type and/or the stimuli:

The NF-κB pathway is involved in regulation of PTX3 production in a model of acute myocardial ischemia and reperfusion in mice (41), whereas induction of PTX3 by HDL requires activation of the PI3K/Akt pathway through a G-coupled lysosphingolipid receptor.

Induction of PTX3 by TNF-α in alveolar epithelial cells is mediated by the JNK pathway.

PTX3 expression can be regulated by the chimeric transcription factor obtained by the fusion of the gene encoding the N terminus of the FUS (fused in liposarcoma) gene in frame to the coding region of the CHOP gene.

The involvement of NF-kB is significant. The dimers in this promoter pathway can be readily activated and thus significant cellular proliferation can occur.

From Stallone et al:

Pentraxins, a superfamily of evolutionary conserved proteins, are essential components of the humoral arm of the innate immune system and play a pivotal role in vascular biology. Pentraxin-

[75] Bottazzi, Barbara . An Integrated View of Humoral Innate Immunity: Pentraxins as a Paradigm (Annual Review of Immunology Book 28) (Kindle Locations 179-193). Annual Reviews. Kindle Edition.

3 (PTX3), the prototype of long pentraxins, differs from short pentraxins for gene organization, cellular source and ligand-binding capacities.

Like short pentraxins, PTX3 facilitates dysregulation of mitogenic signalling pathways, sustains cellular proliferation, angiogenesis, insensitivity to apoptosis, cancer cell invasion and migration, and tumour escape from immunosurveillance.

Unlike short pentraxins, such as C reactive protein (CRP), PTX3 is not produced by hepatocytes but synthesized by a variety of cell types at the site of inflammation, whereby it seems to regulate complement activation. Recent findings suggest an insidious relationship between complement and cancer in terms of cellular proliferation and regeneration as well as angiogenesis. Considering that chronic inflammation is found in as much as 80% of PBxs and the potential role of PTX3 in inflammatory-related carcinogenesis, the aim of present study was to determine whether PTX3 prostate tissue expression and serum levels could predict progression from chronic prostate inflammation to PCa.

From Shishido et al:

Complement products such as C1q, C3, C3a, C4, C5 and the MAC are detectable in the tumor microenvironment . These activated complement proteins have three mechanisms for complement-mediated destruction of tumor cells:

a) complement-dependent cytotoxicity (CDC) ,

b) indirect Ab-dependent, cell-mediated cytotoxicity , which can be complement receptor-dependent and

c) CR3-dependent cellular cytotoxicity (CR3- DCC) , which is relatively rare with tumors.

Complement components are deposited in various tumor types, indicating that activation of complement may contribute to immunosurveillance of malignant cells. Complement proteins C5b-9 are deposited on the cellular surface of breast cancer cells and papillary thyroid carcinoma cells…

Tumor cells have natural mechanisms for self-protection against the complement system, specifically MAC and the cytotoxic activation of CR3. Extracellular protectors such as membrane and soluble complement inhibitors are released by tumor cells into the microenvironment and interfere with complement cascade activation and limit the quantity of complement deposition.

Membrane complement inhibitors, including CD35 (CR1), CD46 (MCP) and CD55 (DAF) control the activation of complement at the level of C3. These serve as an important mechanism of self-protection, making the cells insensitive to the action of complement. Although the complement system regulates inflammation and the innate immune response, complement proteins also aid in tumor growth and immunosuppression. …

Complement anaphylatoxins may alter cellular differentiation resulting in immune suppression. In healthy individuals, myeloid-derived suppressor (MDS) cells differentiate to macrophages, dendritic cells and neutrophils. However, when trapped in the intermediate stage of differentiation, MDS cells may mediate tumor-induced immune suppression. …

These complement stimulated MDS cells also prevent the activation of CD4+ and CD8+ T cells, inhibit Natural Killer cell cytotoxicity, stimulate cytokine production for tumorigenesis and increase angiogenesis. The complement system proteins such as C5a provide inflammatory protection in the tumor microenvironment. Tumor-associated antigens are known to modulate trans-membrane signaling that is required for proliferation, invasion and metastasis of tumor cells.

The presence of NAb against tumor-associated antigens, such as gangliosides of melanoma cells, correlate with increased patient survival . Tumor-reactive Ab exist in healthy wildtype animal blood samples (IgM) and peripheral blood concentrations of NAb increase shortly after initial tumor development and prior to detection of circulating antigens. NAb may have a direct cytotoxic effect on tumor cells, while also inducing a bystander effect during a humoral anti-tumor response.

Thus, NAb recognize tumor-modified cell surfaces that develop during tumorigenesis and activate complement to destroy nascently transformed cells. Tumor cells are able to utilize NAb to escape immunosurveillance.…

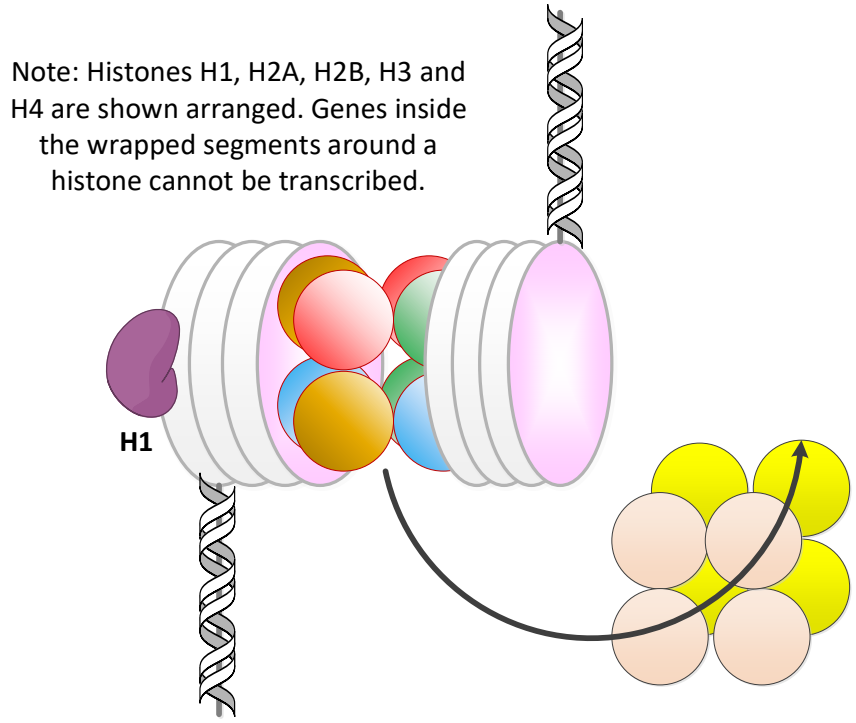
Tumor cells use the innate immune system and NAb to avoid immunosurveillance and elimination. NAb are important for the recognition and elimination of precancerous and cancerous cells. Such tumor-reactive NAb are expressed in multiple tumor types, including melanoma , lung , breast , head and neck and ovarian cancers. …

The humoral innate immune response and NAb specifically have an important role in the recognition and elimination of neoplastic cells.

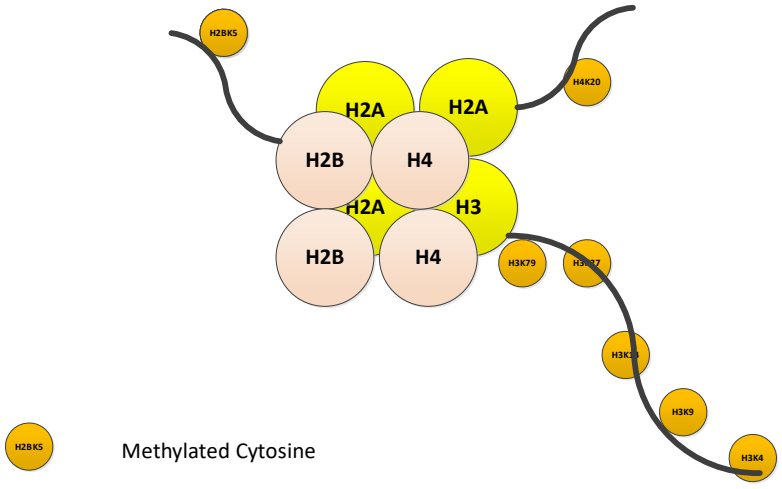
14.2.10Epigenetics and Its Impact

There is an increasing interest in epigenetic influences and cancer. Such effects as methylation result in gene promoter interference and gene expressions. It is not only the methylation of CG islands and the histone methylation and acetylation, but miRNAs and lncRNAs which add up to a significant and complex changing of what the DNA can and should accomplish. There are a plethora of definitions for epigenetics and Dawson et al use one, we shall look at an expanded view where epigenetics represents any effect resulting from the interference of gene expression. Thus the miRNA and methylation and acetylation effects are merely some of the many epigenetic effects. Epigenetic effects are also an important result of inflammation and as a result we will consider some examples to drive the point.

Let us begin with a brief discussion of histones. We demonstrate this below. The histones are a complex of eight proteins around which is wrapped the double stranded DNA. The DNA to be effective must be unwrapped.



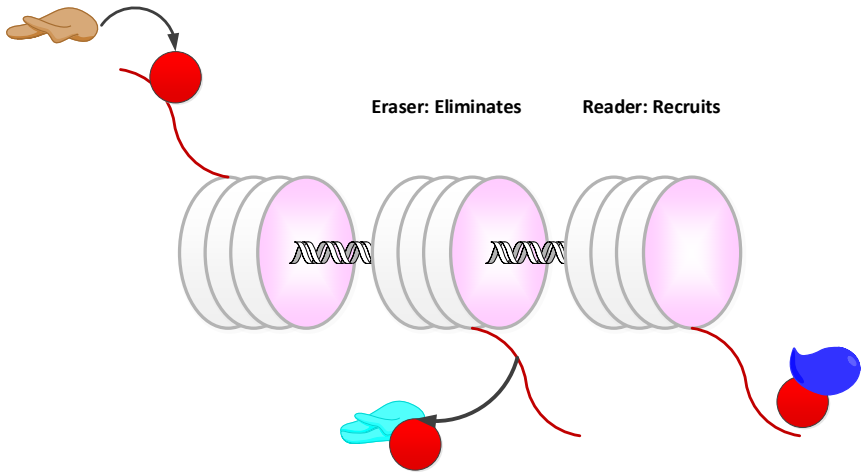
Now the histones unwrap the DNA for expression. The histones have tails and these tails are protein segments that can be methylated or acetylated. The methylated tails would appear as below:



These tails when so methyl/acetyl ated can perform differently than before. Or equally of the methyl or acetyl element is blocked the histone and subsequently the DNA functions differently.

One area of epigenetic effects is the impact of and on bromodomains, specifically BET[76]. BETs can block the acetyl element on a histone tail thus blocking expansion and expression of DNA. The BET can be activated via an inflammatory response. Thus the BET represents an alternative to the previous DNA expression modifications. Namely this is an example of a DNA expression modification via inflammation and in turn via an epigenetic control[77].

From Dawson et al there are proteins which can modify the elements on the histone tails. This can be accomplished in three ways as we graphically depict below:



As Belkina and Denis note:

The bromodomain is a highly conserved motif of 110 amino acids that is bundled into four anti-parallel α-helices and found in proteins that interact with chromatin, such as transcription factors, histone acetylases and nucleosome re-modelling complexes. Bromodomain proteins are chromatin 'readers'; they recruit chromatin-regulating enzymes, including 'writers' and 'erasers' of histone modification, to target promoters and to regulate gene expression.

Conventional wisdom held that complexes involved in chromatin dynamics are not 'druggable' targets. However, small molecules that inhibit bromodomain and extra-terminal (BET) proteins

[76] Bromodomain and extraterminal (BET) proteins: A family of proteins (BRD2, BRD3, BRD4, and BRDT) characterized by tandem bromodomains that interact with acetylated histones and influence gene expression, cell-cycle regulation, and development.

Bromodomains: Regions within proteins capable of recognizing acetylated histones. Proteins containing bromodomains are involved in transcription, DNA repair, replication, and chromosome condensation.

Acetylation: A reaction that results in the addition of a functional acetyl group to an organic compound. Deacetylation is the removal of the acetyl group. Acetylation is a post-translational chemical modification of both histones and nonhistone proteins.

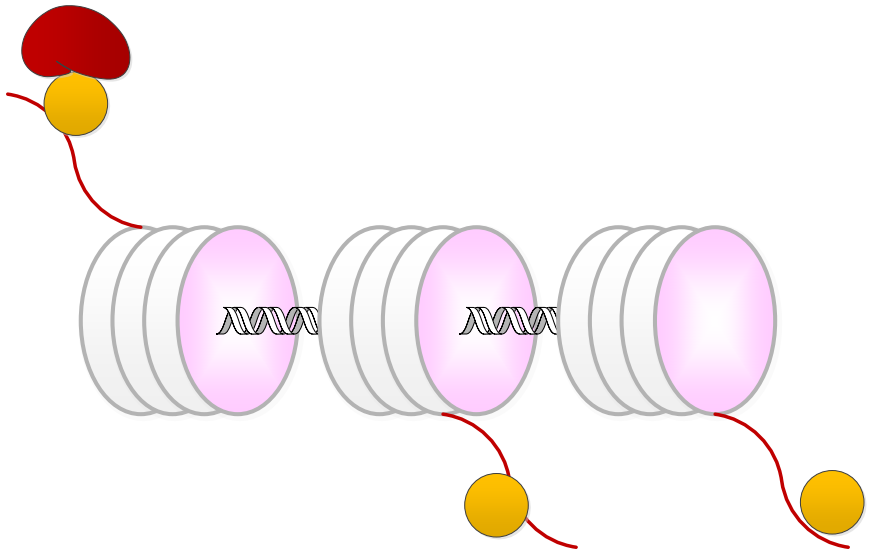
[77] See Gray, Epigenetic Cancer Theory for a discussion. Also see Armstrong, Epigenetics, pp 62-63.

have been described. We examine these developments and discuss the implications for small molecule epigenetic targeting of chromatin networks in cancer.

There is recent evidence that, in addition to BRD2, BRD4 can also co-activate pro-inflammatory genes that depend on NF-κB transcription, through interaction with acetylated RELA. A full account of the interplay of BRD2, BRD3 and BRD4, and how they co-activate NF-κB and cooperate with SWI/SNF complexes to regulate the transcription responses of genes that encode important pro-inflammatory cytokines, such as TNF and IL-6, awaits exposition. These data are potentially relevant to the links between unresolved chronic inflammation or irritation and increased cancer risk, a long-established association. For example, the bowel inflammation that is characteristic of Crohn's disease and related conditions is strongly linked to colorectal cancer.

It is possible that inflammation promotes certain obesity-associated cancers that are resident in or near to inflamed white adipose tissue in insulin-resistant obese subjects. The role of unresolved, chronic inflammation and metabolic dysfunction in obesity-associated cancers is a considerable public health problem, and new epigenetically acting drugs such as the BET protein inhibitors might provide a novel pathway for treating or preventing obesity-associated cancer. Additional preclinical studies are required to more firmly establish the mechanisms underlying hypotheses that the anticancer and anti-inflammatory properties of BET protein inhibitors usefully combine in a chemo-preventive strategy for the obesity-associated cancers.

The bromo domain is that set of 4 protein sequences which is common across the class. This common domain has the ability to attach itself and act as a histone editor as we depicted above. Thus as shown below the BET using the bromo domain can attach to an acetyl element on the histone tail and dramatically change the way genes are then expressed.



The above authors continue:

The mechanistic links between inflammation and cancer, and between inflammation and insulin-resistant obesity, ground an overarching hypothesis that, at the transcriptional level, chronic

inflammation in obesity exacerbates risk for both metabolic complications and cancer. The ongoing and anticipated dire consequences of the world wide epidemic of obesity highlight the translational importance of this discovery, because about 90% of type 2 diabetes is attributable to obesity.

Chemoprevention of obesity-associated cancers by uncoupling NF-κB driven cytokine gene expression with small-molecule BET protein inhibitors would represent an innovative, epigenetically based approach to protect obese subjects who are at risk of both diabetes and cancer. Targeting one set of processes with a BET protein inhibitor might confer benefit by targeting other, apparently orthogonal transcriptional networks that are actually fundamentally related. An extraordinary interconnectedness of chromatin-dependent transcription programmes is thus revealed.

One could argue that the insulin resistant obesity, of which we are seeing epidemic proportions occurring, especially in youth, could be a set up for multiple malignancies. The BET elements may very well be key initiators of the process.

From Sahai et al,

There is increasing interest in inhibitors targeting BET (bromodomain and extraterminal) proteins because of the association between this family of proteins and cancer progression. BET inhibitors were initially shown to have efficacy in hematologic malignancies; however, a number of studies have now shown that BET inhibitors can also block progression of non-hematologic malignancies.

…we summarize the efficacy of BET inhibitors in select solid tumors; evaluate the role of BET proteins in mediating resistance to current targeted therapies; and consider potential toxicities of BET inhibitors. We also evaluate recently characterized mechanisms of resistance to BET inhibitors; summarize ongoing clinical trials with these inhibitors; and discuss potential future roles of BET inhibitors in patients with solid tumors. …

Epigenetic changes that occur during cancer progression are increasingly recognized as a potential target for therapeutic intervention. Bromodomains (BRDs) are evolutionarily conserved protein interaction modules that bind to acetylation motifs present in histones and enable recruitment of transcription factors and other chromatin regulators during the precise sequence of events involved with RNA transcription.

*The BET (**BRD and extra-terminal**) family of proteins regulates the transcription of genes involved in several human diseases and includes family members BRD2, BRD3, BRD4, and the testis-specific BRDT. Significantly, BRD4 has been established as a key regulator of transcriptional elongation by recruiting the positive transcription elongation factor b (P-TEFb) complex to chromatin.*

BRD4 also mediates the formation of the active form of P-TEFb, which in turn phosphorylates and activates RNA polymerase II. BRD4 is enriched in large numbers of enhancer regions, and also in some large super-enhancer regions, and mediates expression of key transcription factors

important for cancer development and progression. BET inhibition displaces BRD4 from these super-enhancers and blocks expression of certain key oncogenes, such as MYC. Besides binding histones, BRD proteins can also regulate cellular function by binding to a number of other proteins.

The following is a list of some of the bromodomain impacts and related malignancies.

Gene in Bromodomain	**Cancer**
KAT3A (CBP) H2AK5 H2BK12–K15 H3K14–K18 H4K5–K8	Acute myeloid leukemia, acute lymphoblastic leukemia, diffuse large B-cell lymphoma, non-Hodgkin's lymphoma, and transitional-cell bladder cancer
KAT3B (p300) H2AK5 H2BK12–K15 H3K14–K18 H4K5–K8	Colorectal, breast, pancreatic, acute myeloid leukemia, acute lymphoblastic leukemia, diffuse large B-cell lymphoma, transitional-cell bladder cancer
SMARCA4 (BRG1)	Lung, rhabdoid, medulloblastoma, breast, prostate, pancreas
SMARCA2 (BRM)	Squamous-cell carcinomas of the head and neck
BRD1 PHD finger	Acute lymphoblastic leukemia
BRD3	NUT midline carcinoma
BRD4 NUT midline carcinoma	NUT midline carcinoma
TRIM33 PHD finger	Papillary thyroid
PBRM1 Renal, breast	Papillary thyroid

For example in Shtivelman et al:

The recent demonstration of preclinical efficacy of inhibiting bromodomain and extraterminal (BET) proteins in different malignancies may be applicable in CRPC. BET domain protein BRD4 was shown to interact with the N-terminal domain of AR, and the BET domain inhibitor JQ1 disrupts AR transcription program in vitro and inhibited growth of CRPC in mouse models in vivo, presenting a new epigenetic approach

Thus the bromo domain is one of many examples where epigenetic effects can give rise to gene expression deviations. These in turn result in malignant expression.

14.2.11Examples

PTX3 has a significant use as a marker in many cancers.

14.2.11.1 Prostate

We have already demonstrated the usefulness in PCa. From Tony et al we have:

The term proteomics was introduced as an analogy to that of 'genomics' [108]. While genomics involves the study of the genes that code for a protein, proteomics is focused on studying the proteins themselves—thus providing a clearer reflection on cellular activity [109]. Proteomic-based experiments can be used to characterize any alterations in protein expression during disease progression.

The emerging field of proteomics has had a tangible impact on biomarker discovery in PCa. A useful cancer protein biomarker would be a protein measurable in body fluids or tissues that could reflect the presence of cancer and provide information on the cancer's stage, aggressiveness and how well the patient is responding to therapy.

For such a biomarker to be clinically applicable, however, it must also meet the following criteria: (i) the protein must be easy to measure at a reasonable cost; (ii) elevated levels of the protein must provide information that would not be available without that protein and (iii) the information obtained from measurement of the protein can be used to guide clinical decision making. Due to the complex nature of cancer, uniformity is non-existent among each histologic cancer type and within each individual tumor. As such, examination of combinations of potential protein biomarkers as panels is believed to provide greater promise for improved PCa diagnosis and monitoring. This trend is reflected in the most recent publications related to PCa associated biomarker discovery

They then include the following Table for a broad summary wherein PTX3 is but one:

Title	Marker(s)
Prostate stromal cell proteomics analysis discriminates normal from tumour reactive stromal phenotypes	Proteins including TAGLN, VDAC1, VDAC2, ALDH1A1
Novel potential serological prostate cancer biomarkers using CT100+ cancer antigen microarray platform in a multi-cultural South African cohort.	41 antigen biomarkers including GAGEI, ROPN1, SPANXA1, PRKCZ, MAGEB1, p53, S15A, S46A, FGFR2, COL6A1, CALM1
Quantitative proteomic study of human prostate cancer cells with different metastatic potentials	SETDB1
Phosphoproteome analysis demonstrates the potential role of THRAP3 phosphorylation in androgen-independent prostate cancer cell growth.	THRAP3
Glycosylation status of serum immunoglobulin G in patients with prostate diseases	Glycosylation changes in IgG
Interlaboratory study on differential analysis of protein glycosylation by mass spectrometry: the ABRF glycoprotein research multi-institutional study 2012	Glycoforms of PSA
An integrative proteomics and interaction network-based classifier for prostate cancer diagnosis	3 proteins (PTEN, SFPQ, HDAC1)
Identification of novel serological tumor markers for human prostate cancer using integrative transcrip tome and proteome analysis	IMPDH2
Identification of phosphorylated proteins involved in the oncogenesis of prostate cancer via Pinl-proteomic analysis	TFC
Urinary CD14 as a potential biomarker for benign prostatic hyperplasia—discovery by combining MALDI-TOF-based biostatistics and ESI-MS/MS-based stable-isotope labeling	CD14
Proteomics-based signature for human benign prostate hyperplasia and prostate adenocarcinoma	15 proteins (TPM1, PHB, KRT8, TUBB2, DES, Glycerol 3 phosphate, P4HB, EHHADH, HSPA5, KRT18, SERPINA1, CKB, HSPA8, ATP5B, ANXA4
CD90/THY1 is overexpressed in prostate cancer-associated fibroblasts and could serve as a cancer biomarker	CD90/THY1
Proteomic analysis of pancreatic secretory trypsin inhibitor/tumor-associated trypsin inhibitor from urine of patients with pancreatitis or prostate cancer	PSTI
In vivo chemoresistance of prostate cancer in metronomic cyclophosphamide therapy	3 proteins (TXN, CTSB, ANXA3)
The cancer-related Runx2 protein enhances cell growth and responses to androgen and TGF-beta in prostate cancer cells	Runx2
Proteomic analysis of conditioned media from the PC3, LNCaP, and 22Rvl prostate cancer cell lines: discovery and validation of candidate prostate	4 proteins (FST, CXCL16, PTX3, SPON2)

14.2.11.2 Lung

In the work by Diamandis et al:

PTX3, but not KLK11 or progranulin, is a new serum biomarker for lung carcinoma. Its diagnostic sensitivity and specificity is similar to other clinically used lung cancer biomarkers.

More studies are needed to establish if PTX3 has clinical utility for lung cancer diagnosis and management.…We conclude that PTX3 is a novel biomarker for lung carcinoma, which displays comparable sensitivity and specificity to other currently used lung cancer biomarkers. It appears that the observed serum elevations of PTX3 are associated with inflammation and cancer cell apoptosis around the tumor microenvironment.

More studies will be necessary to establish if PTX3 has clinical utility in lung cancer, either alone or as a member of a biomarker panel. In such studies, inclusion of patients with benign lung diseases, in order to further assess the specificity of these biomarkers, would be important. As mentioned earlier, PTX3 may also be elevated in other malignancies such as prostate cancer and liposarcomas.

14.2.11.3 Head and Neck

From Chang et al:

Overexpression of the epidermal growth factor (EGF) receptor (EGFR) is associated with enhanced invasion and metastasis in head and neck squamous cell carcinoma (HNSCC). Long Pentraxin PTX3 is involved in immune escape in cancer cells. Here, we identified PTX3 as a promoting factor that mediates EGF-induced HNSCC metastasis.

EGF-induced PTX3 transcriptional activation is via the binding of c-Jun to the activator protein (AP)-1 binding site of the PTX3 promoter. PI3K/Akt and NF-kB were essential for the PTX3 activation. EGF-induced PTX3 expression was blocked in c-Jun- and NF-KB-knockdown cells. EGF-mediated PTX3 secretion resulted in the enhancement of cell migration and invasion, and interactions between cancer and endothelial cells.

The tail-vein injection animal model revealed that depletion of PTX3 decreased EGF-primed tumor cell metastatic seeding of the lungs. In addition, fibronectin, matrix metalloproteinase-9 (MMP9) and E-cadherin were essential components in EGFR/PTX3-mediated cancer metastasis. In conclusion, PI3K/Akt and NF-KB-dependent regulation of AP-1 mediates PTX3 transcriptional responses to EGF. Autocrine production of EGF-induced PTX3 in turn induces metastatic molecules, activating inflammatory cascades and metastasis.

14.2.11.4 Pancreas

From Kondo et al:

Pentraxin family members, especially PTX3, may be used as promising biomarkers in the prognosis of pancreatic carcinoma patients.…Inflammatory responses have decisive roles at different stages of tumour development, including initiation, promotion, malignant conversion, invasion, and metastasis, and affect immune surveillance and response to therapy. The invasive capacity of malignant cells has been observed to increase in the presence of inflammatory cytokines, including TNF-alpha, interleukin (IL)-1beta, and IL-6, as well as transcription factors, including AP-1, NF-kB, and STAT3.

In a previous study, we identified C-reactive protein (CRP), which is produced via IL-6 and TNF-alpha stimulation in the liver, as an important factor in the prognosis of pancreatic carcinoma. In other studies, long pentraxin (PTX3), a member of the pentraxin family, which includes CRP and whose members may have a significant role in tumour inflammatory and malignant behaviours, was reported to be overexpressed in several malignancies, including liposarcomas and lung cancer.

These findings indicate that reduction of the key inflammatory mediators may be an important means of promoting antitumour activity. In a previous study, we had observed direct secretion of PTX3 from pancreatic carcinoma cell lines in vitro. Building on this finding, we aimed to determine the biological significance of PTX3 in pancreatic cancer via further in vitro study of several pancreatic carcinoma cells lines, as well as prospective clinical investigation of the clinical significance of plasma PTX3 expression in chemotherapy naive pancreatic carcinoma patients. We found that PTX3 expression might be a promising biomarker for pancreatic carcinoma prognosis

14.2.12Observations

We have examined the issue of inflammation and the impact on the immune system and cancer. These have been issue of concern for at least a century. We know how powerful the immune system can be, both for good and for adverse events. Inflammation also has been a major concern and we are just beginning, in my opinion, to understand its causes and effects. Obesity is a major problem, and within the context of obesity is the issue of inflammation, especially for those who have Type 2 Diabetes. Thus there is a clear need to understand inflammation, especially as we are able to deal with many cancers, and most likely cancer may then become a co-morbidity of a high costs for those who are obese.

In this paper we have used a rather idiosyncratic approach to the subject. On the one hand we focus on the innate immune system, and specifically pentraxins. Secondly we look at pentraxin and in contrast BET, bromodomain, effects and cancers. The reason why is simply that both relate directly to inflammation and specifically obesity. Thirdly we examine these across a broad spectrum of organs and finally we have included some discussions on the free radical problem. They all fit nicely since free radicals are a result of obesity and Type 2 diabetes, the resulting inflammation is chronic, the chronicity drives the innate system via pentraxins and the epigenetic elements via bromodomain proteins. The next result is a dual assault on genes, DNA expression and major epigenetic defects via histone acetyl defects.

The innate system, complement and pentraxins, has been proposed as a means to attack cancer especially in an inflammatory environment. As Macor and Tedesco note:

Direct killing of tumor cells by the membrane attack complex (MAC) represents one of mechanisms used by the C(omplement) system to control tumor growth. However, C may also exert its antitumor activity through additional non-cytotoxic effects. Thus C3b deposited on tumor cells and subsequently converted into iC3b promotes binding of these cells to the C receptors CR1 and CR3 expressed on human leukocytes. Although CR1 and CR3 fail to trigger the killing of tumor cells following their interaction with their respective ligands, C3b and iC3b,

evidence collected both in vitro and in vivo indicate that the adhesion of iC3b-coated tumor cells to phagocytes and natural killer (NK) cells expressing CR3 (CD11b–CD18) results in C-dependent cell cytotoxicity (CDCC) provided that a second signal is delivered to tumor cells by anti-tumor Abs (Fc-FcR) that mediate Ab-dependent cellular cytotoxicity (ADCC).

These data suggest that the C system plays an important role in immunotherapy of cancer and acts as an additional weapon in support of the standard therapy provided by surgery, chemotherapy and radiation against tumor cells particularly in the control of the minimal residual disease. Optimal conditions are required for C to be effective, which include the level of expression of tumor antigens present on the surface of tumor cells, the class of Abs and the reduced expression of C inhibitors.

These data suggest that the C(omplement) system plays an important role in immunotherapy of cancer and acts as an additional weapon in support of the standard therapy provided by surgery, chemotherapy and radiation against tumor cells particularly in the control of the minimal residual disease. Optimal conditions are required for C to be effective, which include the level of expression of tumor antigens present on the surface of tumor cells, the class of Abs and the reduced expression of C inhibitors.

We can thus ask if we have a cause and effect and if so do we have a control mechanism. As we have seen great strides in immunotherapy via PD-1 and like Mabs and controls we could anticipate a similar result with the targeting of the innate and epigenetic drivers.

15 CONCLUSIONS

We have attempted to present cancer immunotherapy as a potential study in system thinking. The difficulty is that it is a field in continual flux, one where new elements are being discovered daily and when some approach is applied we find what works and what does not. It is often in seeing what does not work that we obtain new insights.

Our intent was to lay out with what is known at present, and with just some basic fundamentals at that, and suggest a process to see immunotherapy as a systems problem. Namely we can understand elements, and we can understand the input/output relationships and then we can utilize principles in uncertain systems to seek control mechanisms alongside of identification processes. We also try to lay out the ever growing list of "tools" which can be brought to bear on this problem.

On the down side of this approach we have the concern that the immune system is a very powerful system. If not controlled properly we can see it play out almost in a carpet bombing manner setting loose a devastation of the very person who we are trying to save. This has happened on multiple and repeated occasions.

At the other extreme we have many results which do not comply with standard theory and we do not see them published for a lack of an explanation. Two come to mind. One is the CIK applications in MDS where using the homologous stem cell transplant cells to increase their efficacy by using CIK, instead of the homologous cells taking over we see the patients original immune system kick in aggressively and come back in a fully recovered manner. Why? Good guess. The second is the HG PIN, a cancer in situ of the prostate. There are a set of samples where there were patients with considerable PIN which one would have expected to go to PCa, but upon subsequent biopsies the prostate is totally benign. Are these immune system reactions? If so, what caused them and how do they work. Often the very fact that we cannot explain a mechanism leaves the observation hidden.

15.1 SUMMARY OF OPTIONS

As a means to review and place in context what we have presented herein we will return to the work of Harris and Drake and examine their proposed structure for understanding cancer and immunotherapy. They categorize the opportunities as follows:

(1) Dendritic cells: Dendritic cells (DC) link the innate immune system to the adaptive immune response. These cells dwell in the tissues, continually sampling the microenvironment and taking up antigens primarily through pinocytosis. When the innate immune system is activated in their vicinity, DCs sense this as “danger”, cease antigen uptake and travel to local lymph nodes, where their role is to present antigen to specific T lymphocytes. The microenvironment in which a DC acquires antigen determines whether the DC will have the capacity to activate an antigen-specific lymphocyte or to tolerize the lymphocyte. In addition to pinocytosis, immature DCs are also How can our knowledge of DC biology be used to develop immunotherapy for patients? While it is clear that activation of these cells is desirable, there are two general approaches to

achieve that end: ex vivo and in vivo activation. Ex vivo strategies for DC-based immunotherapies include generation of DCs from circulating monocytes via subsequent culture, as well as procedures in which DCs are derived from circulating CD34+ hematopoetic stem cells (HSCs).

In the U.S., an immunotherapy based on ex vivo activated DCs has been FDA-approved to treat patients with metastatic prostate cancer. This product, sipuleucel-T is generated by incubating a patient's monocytes with a fusion protein that links the target antigen (Prostatic Acid Phosphatase) to the cytokine GM-CSF; here GM-CSF serves to mature the monocytes toward DCs, and assists in internalization of the antigen. After ex vivo incubation, the mixed cellular product, including maturing DCs, is re-infused into patients.

In a randomized control trial for prostate cancer patients, this product resulted in a 4.1 month improvement in median survival compared to placebo. Another common strategy for DC-based immunotherapy involves maturation of immature monocytes into DCs by culturing them for several days in the presence of GM-CSF and IL- 4. The DCs are then loaded with tumor-specific peptides or in some variation with whole protein antigens which they must subsequently process and present. While ex vivo stimulation of DCs often results in quantifiable immune and clinical responses with no dose limiting toxicities, the overall clinical response rates to this therapeutic approach have remained somewhat low.

Dendritic cells are scavengers. They just wander around picking up information on what may be hanging around that should not be. The use of them as noted above for PCa shows a 4.1 month improvement in survival. One wonders however if 4.1 months is worth the cost of the therapy which can exceed $100,000. This is similar to many other similar immunotherapies. Dendritic cells are a somewhat strong and powerful method which may not be that specific and not that efficacious.

(ii) Antibodies as therapy: Monoclonal antibodies are now widely utilized in the treatment of a number of tumor types; pertinent examples including trastuzumab (anti-Her-2) for the treatment of breast cancer, rituximab (anti-CD20) for the treatment of lymphoma, and the recently approved immunoconjugate T-DM1, which fuses trastuzumab to a highly potent chemotherapy, emtansine (DM1 [deacetyl maytansine]) to facilitate local delivery and minimize systemic toxicity. Antibody-based immunotherapeutics can be exquisitely specific treatment tools, based on the diverse and nanomolar level affinity of the Fv region of the antibody for its target, as well as the ability of the Fc region to engage components of the host immune system.

How do monoclonal antibodies work? The mechanisms of action of unconjugated monoclonal antibodies include blocking a pro-survival signal, as well as facilitating tumor cell destruction by the binding of the Fc portion of the antibody to Fc Receptors on natural killer (NK) cells—promoting the ability of NK cells to lyse their targets through a process known as antigen-dependent cytotoxicity (ADCC).

Monoclonal antibodies can also mediate cytotoxicity by binding to complement receptors on effector cells, a process known as complement-dependent cytotoxicity (CDCC). The Fc portion of a monoclonal antibody plays a major role in determining the immune mechanisms induced, with

monoclonal antibodies of the human IgG4 isotype primarily functioning as "blockers". One interesting aspect involved in the development of monoclonal antibodies for the clinic involves their affinity, while higher antibody affinity results in increased target engagement and ADCC, higher affinities can also result in decreased tumor penetration and compromised efficacy

Mabs seem to be everywhere and have gotten to the point of being somewhat specific and efficacious. The techniques for developing, testing and producing them drives down the costs and improves the efficacy. However, they have yet to show significant results in some cancers. Ipilimumab is an example in metastatic melanoma as we discussed.

(iii) T cell intracellular signaling: To understand how the adaptive arm of the immune system is engaged, a basic knowledge of T cell biology and activation can be helpful. T cells detect antigen bound to MHC molecules, with the CD4+ T cell subset binding to MHC Class II primarily expressed on APCs while CD8+ T cells are activated by binding to MHC Class I, which can be expressed by APCs as well as normal cells.

Following initial APC-driven activation, CD8+ T cells may later recognize target cells expressing their cognate antigen, resulting in cell-mediated cytotoxicity. For T cells to be fully activated, the APC must provide other signals in addition to the peptide/MHC (signal 1). The B7-CD28 interaction, with B7 expressed on the APC and CD28 on T cells, was one of the first co-stimulatory signaling pathways elucidated.

T cells are powerful tools to attack specific cells. They generally can be fine-tuned to perform the task depending on how well we understand what must be targeted.

(iv) Memory T-cells: Following initial activation, a minority (5-10%) of T cells become long-lived memory cells with enhanced functional responses upon antigen re-encounter as compared to naïve T cells. For cancer immunotherapy the importance of generating functional memory cells is two-fold. First, the presence of memory cells could potentially decrease metastatic spread and prevent tumor re-growth after an initial response. Second, memory cells could limit de novo induction of a second malignancy.

The importance of tumor infiltrating memory T cells is further illustrated with the novel Immunoscore, which has demonstrated prognostic and predictive value in colorectal cancer through the quantification of tumor infiltrating cytotoxic effector cells and memory T cells. Current understanding of memory T cells is derived largely from the study of the immune response to microbes; however, in the absence of good models of memory induction in tumor bearing animals or humans, one can reasonably extrapolate these findings to anti-tumor responses.

Targeting specific antigens is also a significant tool.

(v) Tumor antigens and immunogenicity T cells recognize antigen in the form of small peptides, derived from proteolysed substrates, and presented in the context of MHC molecules. MHC molecules are genetically diverse, and for each MHC variant, only specific peptide sequences from a given antigen are able to bind for presentation to T cells and subsequent induction of

anti-tumor immune responses. Understanding the specific antigens recognized by the immune system and the specific peptide sequences presented on MHC can be important in improving immunotherapies directed against a specific antigen. Several approaches have been used to identify tumor specific antigens, including molecular cloning; sequencing of antigenic peptides; and computer algorithms, each of which has its relative benefits and deficiencies.

The CAR approach is an extreme in specific targeting which has seen applicability to hematological cancers.

(vi) Adoptive cellular therapy: Adoptive T cell therapy allows for ex vivo stimulation of lymphocytes in a non-tolerizing environment followed by re-infusion of activated T cells into patients. There are varying sources and types of T cells used for adoptive therapy, these include tumor infiltrating lymphocytes (TILs), T cells engineered to express a cancer-specific TCR, and T cells engineered to express a chimeric antigen receptor (CAR) that combines the extracellular portion of an antibody with the T cell receptor signaling machinery.

Of these approaches, expanded TILs are the least labor intensive to produce, yet require an invasive procedure to obtain. Additionally, maintenance of TILs after adoptive transfer usually requires high dose IL-2, which results in significant toxicity. Clinical response rates in patients with metastatic melanoma treated with expanded TILs is impressive, approximately 50% in several studies

Thus there is an ever expanding set of immunotherapeutic tools available to attack the cancer cells.

15.2 Risks of Therapy

There are many risks related to immunotherapy. As Baldo notes:

Although mAbs used for cancer immunotherapy are generally better tolerated than widely used 'conventional' chemotherapeutic agents, adverse events following the administration of mAbs can result from a variety of mechanisms and may be quite diverse.

The wide variety of mAb associated reactions ranges from, for example, headache, mild gastrointestinal symptoms such as diarrhea, transient rash and itching to severe cytopenias, cardiac toxicity, anaphylaxis, exfoliative dermatitis and rarely life-threatening bullous toxidermias.

Being non-endogenous proteins of sufficient size, immunogenicity is always a safety concern and despite progressive efforts in developing chimeric, humanized, and fully human mAbs, the possibility of generating anti-idiotype antibodies means that the potential immunogenicity of mAbs persists, at least to some degree.

Although ADRs such as anaphylaxis, serum sickness, autoimmune diseases, urticaria, Stevens-Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN) are clearly mediated by the

immune system, others like some cytopenias (thrombocytopenia, neutropenia and anemia) may or may not be so.

Some pulmonary and liver toxicities, induced infections and cutaneous responses are often less well defined and understood and may have at least an indirect connection to immunological processes. This might also be said about IRs/CRS and the systemic inflammatory response syndrome (SIRS), but the tumor lysis syndrome (TLS) and some cytopenias, as well as heart, pulmonary, hepatic, kidney, embryo-fetal, and neurological toxicities appear to be due to direct cytotoxic actions and/or a number of other non-immune mechanisms.

Baldo summarizes this in the following Table. Note that the extent of responses is quite extensive.

Drug	***Systemic***	***Cutaneous***
Catumaxomab	Abdominal disorders; pyrexia; cytopenia; hepatotoxicity; dyspnea; infections; immunogenicity	rash; erythema; hyperhidrosis; pruritus; allergic dermatitis
Ibritumomab tiuxetan	Infections; severe cytopenia; immunogenicity; secondary malignancies	bullous dermatitis; exfoliative dermatitis
Tositumomab-I131	Anaphylaxis; severe cytopenia; fetal harm; hypothyroidism; secondary malignancies; infection; immunization	In clinical trial: Skin reactions, all grades rash 17%, pruritus 10%, sweating 8%. Grades 3 and 4 - 0 - < 1%; exfoliated dermatitis
Brentuximab vedotin	cytopenia; TLS; immunogenicity; PML; fetal harm; anaphylaxis	SJS; rash; pruritus; alopecia
Cetuximab	cardiopulmonary arrest; GI; pulmonary toxicity; electrolyte imbalance; infection; anaphylaxis	acneiform rash; nail changes; pruritus; xeroderma; paronychial inflammation
rituximab	renal toxicity; infections; cardiac events; pulmonary events; bowel obstruct. and perforation; neutropenia; anaphylaxis	Paraneoplastic pemphigus; lichenoid dermatitis; vesiculobullous dermatitis
alemtuzumab	cytopenia; infections; immunogenicity; cardiac events; pulmonary events	Urticaria; rash; erythema; pruritus

Drug	***Systemic***	***Cutaneous***
Bevacizumab	GI perforation; hemorrhage; wound healing complications; thrombosis; hypertension; necrotizing fasciitis; proteinuria; pulmonary events	exfoliative dermatitis; alopecia
Pertuzumab	embryo-fetal toxicity; cytopenia; GI; PN; hypersensitivity/anaphylaxis	alopecia; rash; paronychia; pruritus
Trastuzumab	Cardiomyopathy; embryo-fetal toxicity; pulmonary events; neutropenia; anaphylaxis/angioedema; anemia; GI	rash; nail disorders; pruritus
Trastuzumab emtansine	Hepatotoxicity; fetal harm; pulmonary events; thrombocytopenia; neurotoxicity; hypersensitivity	rash; pruritus
Denosumab	Hypocalcemia; embryo-fetal toxicity; ONJ and osteomyelitis; fatigue/asthenia; dyspnea	Dermatitis; eczema; rash; pruritus
Ipilimumab	diarrhea; fatigue	Dermatitis; pruritus; rash
Ofatumumab	cytopenia; intestinal obstruction; PML; pneumonia; pyrexia; infections; cough; dyspnea; diarrhea; fatigue	rash; urticaria; hyperhidrosis
Panitumumab	pulmonary fibrosis; pulmonary embolism, electrolyte depletion; GI; fatigue	rash; dermatitis ‘acneiform’; exfoliation; erythema; pruritus; xerosis; paronychia; skin fissures; photosensitivity

For prostate cancer Gao et al present some detailed specific results of ipilimumab usage. The Adverse Events, AEs, include:

The spectrum of irAEs (immune related Adverse Events) ranges from

common reactions, such as dermatitis, colitis, hepatitis, and hypophysitis, to rare conditions such as uveitis, neuropathy, and lupus nephritis.

Overall, irAEs occur in more than 70% of patients treated with ipilimumab. There is a direct correlation between ipilimumab dose and irAE frequency and grade. The majority of irAEs emerge during the first 14 weeks of therapy, although late irAEs can occur as well In patients

treated with the standard dose of ipilimumab (3 mg/kg intravenously [IV] every 3 weeks for a total of 4 doses), irAEs of any grade occurred in about 60% of patients.

The most common irAEs affect the skin (rash/pruritus, about 40%), gastrointestinal tract (diarrhea/colitis, about 30%), endocrine system (5–8%), and liver (about 3%). Grade 3–4 irAEs occur in 6–13% patients, predominantly in the gastrointestinal tract (5–8%), endocrine system (1–4%), and skin (1%).

For CAR-T cell therapy there can be a broader spectrum. As Bonifant et al note:

T cells can be genetically modified to target tumors through the expression of a chimeric antigen receptor (CAR). Most notably, CAR T cells have demonstrated clinical efficacy in hematologic malignancies with more modest responses when targeting solid tumors. However, CAR T cells also have the capacity to elicit expected and unexpected toxicities including: cytokine release syndrome, neurologic toxicity, "on target/off tumor" recognition, and anaphylaxis. Theoretical toxicities including clonal expansion secondary to insertional oncogenesis, graft versus host disease, and off-target antigen recognition have not been clinically evident. Abrogating toxicity has become a critical step in the successful application of this emerging technology. To this end, we review the reported and theoretical toxicities of CAR T cells and their management.

Namely CAR-T cells can have what has been called a "carpet bombing" effect. That results from two areas. One is the "off target" response and the second is the "cytokine storm" effect. One suspects that the adverse events from immunotherapy can be significant and will remain so until improved specificity of targeting is achieved.

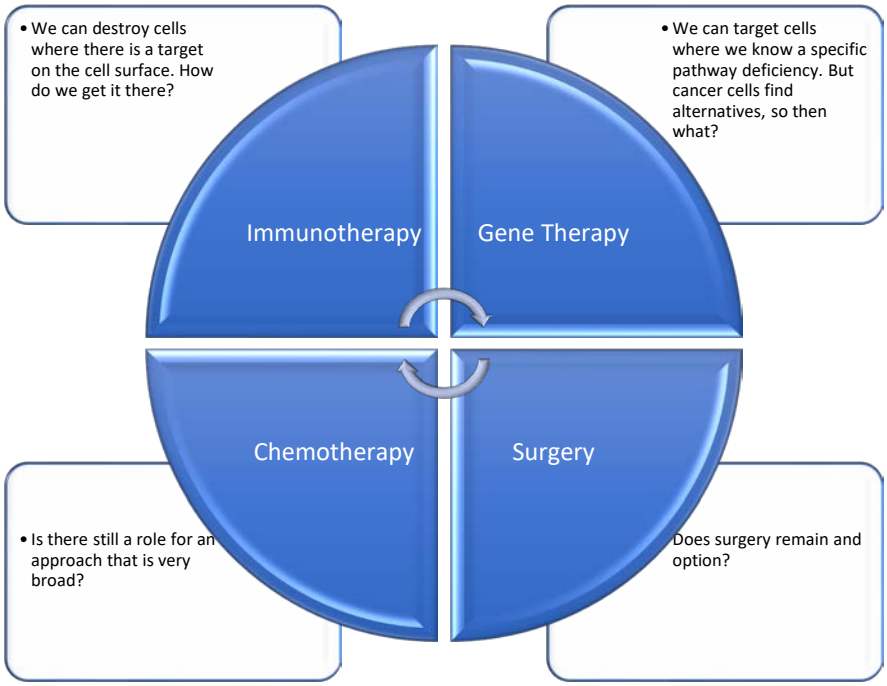
15.3 Integration of Methods

Immunotherapy is but one leg of the tools upon which cancer can be managed. At the other end is an understanding of what genetic changes have occurred in a cell and then to target the pathways which have been activated or suppressed due to that change. For example, in CML we have a transposition, the movement of a gene from one chromosome to another. This led to the classic Philadelphia chromosome. We also have in melanoma the BRAF V600 gene and its suppression. The question we can then pose is:

"How does one best utilize the immunotherapeutic methods with those which utilize the knowledge of gene changes resulting in aberrant expressions?"

Somehow these have become two competing schools of thought. Clearly the immune response has the ability to "attack" and bad cell if we know what differentiates what is on the surface of the cell. However, can we use our tools to get into the pathways of aberrant cells and have the cell put a unique marker on its surface? That, for example, would be a way whereby an immunotherapy could work.

We present four options with some key questions in each as in the below Figure.


• We can destroy cells where there is a target on the cell surface. How do we get it there?
• We can target cells where we know a specific pathway deficiency. But cancer cells find alternatives, so then what?
Immunotherapy
Gene Therapy
Chemotherapy
Surgery
• Is there still a role for an approach that is very broad?
Does surgery remain and option?

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