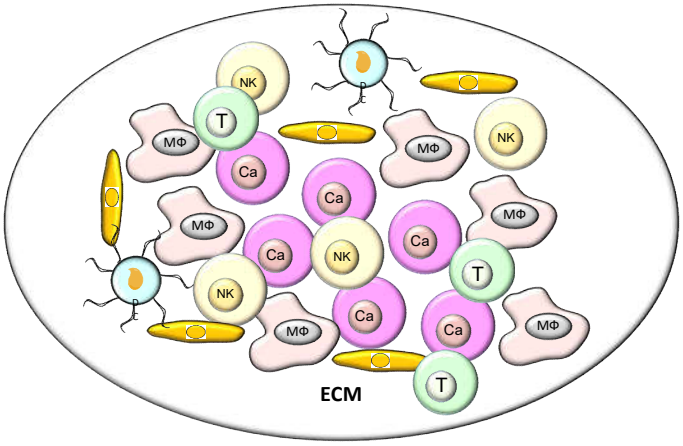


CANCER VACCINES

TGL 206

ABSTRACT

There has been an extensive amount of work examining and developing cancer vaccines. However, the term itself can at times be confusing, for unlike classic vaccines which target pathogens using the immune system, many cancer vaccines target the cancer cells themselves, the effect of the pathogen, if you will, rather than the cause. We examine some of the current approaches.

Terrence McGarty

February 2024

NOTICE

This document represents the personal opinion of the author and is not meant to be in any way the offering of medical advice or otherwise. It represents solely an analysis by the author of certain data which is generally available. The author furthermore makes no representations that the data available in the referenced papers is free from error. The Author also does not represent in any manner or fashion that the documents and information contained herein can be used other than for expressing the opinions of the Author. Any use made and actions resulting directly or otherwise from any of the documents, information, analyses, or data or otherwise is the sole responsibility of the user and The Author expressly takes no liability for any direct or indirect losses, harm, damage or otherwise resulting from the use or reliance upon any of the Author's opinions as herein expressed. There is no representation by The Author, express or otherwise, that the materials contained herein are investment advice, business advice, legal advice, medical advice or in any way should be relied upon by anyone for any purpose. The Author does not provide any financial, investment, medical, legal or similar advice in this document or in its publications on any related Internet sites.

Furthermore, this document contains references to and quotes and modified charts and figures from papers and documents under the premise of "Fair Use" in order to present ideas and understandings in context. The Author has attempted to make any and all references to such material separate from those of the author per se and has referenced the source expressly in all cases. These documents are for the dissemination of ideas and have no commercial intent. Our approach herein is to take elements of what is recent in the literature focused on a specific topic and attempt to develop a tapestry image of these connectable elements. We do not necessarily provide any new or fundamental results but merely attempt to assemble elements in a systematic and holistic manner.

Communications relating to these documents and these should be sent to:
mcgarty@alum.mit.edu.

Access to prior Technical Notes can be made via Research Gate,
https://www.researchgate.net/profile/Terrence-Mcgarty/research

Contents

1 INTRODUCTION

Cancer vaccines have been considered for several decades. We will examine some of the key insights as they are known today and attempt to examine the potential for a cancer vaccine. As we shall note, vaccines can be considered pre-diseased and post-diseased. The now classic pre-disease cancer vaccine is the one for HPV. This vaccine is fundamentally a viral targeted vaccine and the virus is the cause of the cancer, cervix and head and neck. It does not directly affect the cancer but it targets the cause of the cancer, a virus. Thus, like polio, the vaccine inhibits the polio virus and it is the virus that results in polio.

1.1 THE PARADIGM

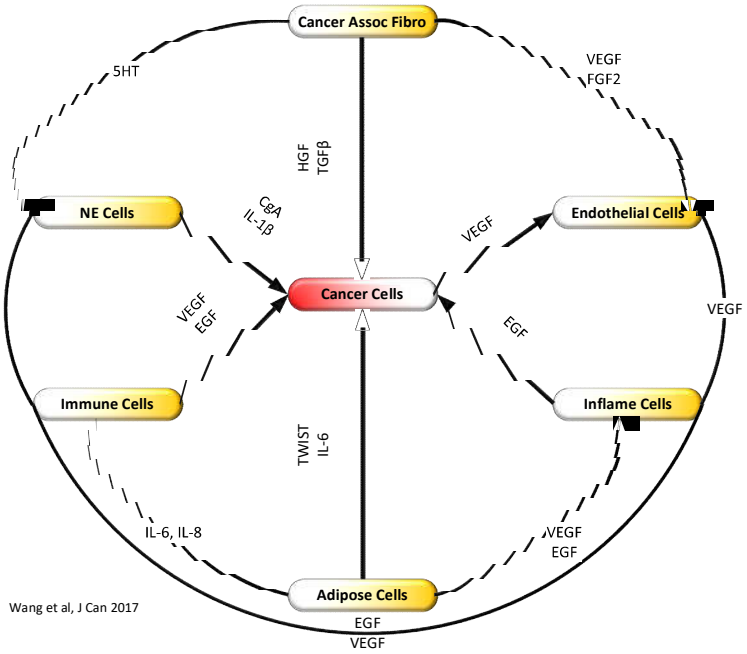
Vaccines have had a classic paradigm, one which we term the **exogenous paradigm**. Namely the pathology is driven by some external factor such as a virus, a bacteria, or a fungus. The attack is from some external source. That source has some identifiable antigen, a protein or likewise, for which was to activate the immune system. When activated by a vaccine the immune system will be prepared to attack the invader if and when it appears. For example, we have smallpox, polio, and of course COVID.

In contrast, one could see cancers as an **endogenous paradigm** attacker. Its paradigm is a bit different. Here the pathology is a modification of a normal cell. It may have some unique or even common antigen target such as a surface protein. One can then develop a vaccine to attack the cancer cells by identifying the Ag, know or to be determined, and then anticipate that the immune system will perform against the cancer cell as it would perform against say a viral particle. Namely activate cells such as T or NK and liquidate the cancer cells identified.

In contrast, the more general concept of a cancer vaccine is an a posteriori approach wherein the cause drive may very well be unknown yet the cancer cell may be identifiable and we use the immune system to attack the aberrant cancer cells. Unlike the exogeneous pathogen approach as first noted the cancer vaccine approach uses the immune system but it does so by targeting specifics of the now known cancer cell.

Both approaches use the immune system to make the attacks. However, the a posteriori approach must deal with the complex tumor micro environment, TME, that complex of the tumor cells and supportive cells, including morphed immune cells themselves. The TME can be a protective coat that even if we can identify unique cancer cell antigens, the immune system must break through the supporting TME. Thus, unlike simple virus vaccines, the cancer vaccine needs to deal with a highly complex environment. It must break through the fog of the TME. Moreover, it must be sustainable. We know that simple viral vaccines can lose efficacy is short periods. COVID in 6 months, rabies in three years yet there are some which can last a lifetime such as small pox. The reasons for this are not fully understood.

Now cancer cells also live in an environment composed of many supportive cells. We demonstrate some of these below. Each of the environmental cells communicate ack and forth with the cancer cell allowing for a complex set of interactions which sustains the malignancy.



Our objective herein is to review some of the current understandings of cancer vaccines and their progress. In addition, we use this example as a means to examine the paradigms used in various cancer therapeutics. Cancer is a highly complex biological process. It extends well beyond the simple construct of a gene mutation. We try to start an examination of that issue. Furthermore the therapeutic options are now many (see Karp et al) and for the most part the newer ones, targeted and immuno therapy, have shown significant results. They are based upon an existing paradigm of targeting a single genetic expression, such as HER2 in breast cancer or PD-1 in many other cancers. Vaccines used as therapeutics attempt to combine targeting with immunotherapeutics. Yet vaccines rely on existing paradigms, namely surface antigens. However, as we discuss, the surface targets are multiple, often the best are yet unknown and vary from cell to cell and patient to patient. In addition, the cancer cells appear as “fur balls” of thousands of ligands, receptors, and peptides. How best to model this, address this, and clinically utilize this is yet to be adequately presented. Namely we will need a new paradigm to seek the best results. Thus, our objective is to raise this issue and not to answer it.

1.2 Some History

We can now examine two snapshots in the development of cancer vaccines. First from Gilboa in 2004 it states:

As discussed above, the tumour microenvironment is not conducive to the emigration and optimal activation of DCs, so the ensuing immune response is weak and ineffective. The purpose of specific active immunotherapy is to stimulate an antitumour immune response by channeling the tumour antigens into the appropriate DC subset and providing the optimal conditions for the maturation of the DC into a potent immunostimulatory APC.

There are four important issues to consider in designing effective cancer vaccines:

1. how to identify potent tumour rejection antigens;

2. how to stimulate an effective antitumour immune response;

3. how to avoid autoimmune pathology; and

4. how to prevent immune evasion. …

The above four objectives remain constant. However, understanding things such as the TME and methylation may also be essential. They continue:

Identifying tumour-rejection antigens.

Tumour antigens have been isolated from cDNA libraries or deduced from peptides that are eluted from the surface of tumour cells by virtue of their being recognized by tumour specific cytolytic T cells. The potency of tumour antigens depends on the frequency and avidity of the corresponding T cells that are present in the patient's T-cell repertoire. Both variables are largely determined by the extent of tolerance that is triggered against the antigens. The importance of T-cell avidity for biological function is well documented. Yet the activation of low-avidity T cells, which can be accomplished by using increasingly effective immunization protocols, can also elicit an effective antitumour response

With this in mind, tumour antigens can be divided into two main categories: patient specific mutated self-antigens and shared non-mutated self-antigens. Mutated self-antigens result from somatic mutations in normal gene products, reflecting the genetic instability of tumour cells.

The above was a remains a key observation. However, it is important to know what cells contain what antigens. It becomes a massive task. The stem cell issue may come to play. As we shall see, the current approach may target dozens of antigens. Is that because there are dozens of different cells or really dozens on all cells?

By and large, such mutations have arisen in a random fashion and are incidental to the oncogenic process. Therefore, tumour antigens in this group will be patient-specific, not expected to trigger tolerance and should make potent tumour-rejection antigens. As the identification and isolation of mutated self-antigens from each cancer patient, although technically feasible, is not practical, the alternative option is to vaccinate patients with autologous tumour-derived antigenic mixtures. Animal studies indicate that this approach, despite the small proportion of relevant tumour antigens in the mixture, is a powerful method to stimulate antitumour immunity. A general limitation of this approach, however, is that it will not be possible to obtain sufficient tumour tissue or tissue of sufficient purity from many patients for antigen preparation.

Use of mRNA-encoded antigens, which can be amplified from small amounts of tumour tissue, could overcome this logistical hurdle.

A fundamental concern underlying vaccination with autologous antigenic mixtures is that the antigenic profile of the progressing tumour is bound to change with time49,50 and, therefore, the immune response that is elicited during immunization could be directed against antigens that are long gone. Vaccination with defined and well-characterized shared antigens is clearly the method of choice.

However, as shared antigens (with a few notable exceptions) correspond to normal gene products, many antigens belonging to this group will have triggered tolerance to varying degrees and vary accordingly in their effectiveness as targets for immunotherapy. Tumour antigens corresponding to fetal gene products or products that are expressed in immuno-privileged sites, such as carcinoembryonic antigen or MAGE-family antigens, will have triggered little or no tolerance and should make excellent tumour-rejection antigens, whereas tissue specific products such as MART1, SILV (also known as gp100) or ERBB2 (also known as HER2/NEU) are likely to have triggered some degree of tolerance and would make weaker tumour-rejection antigens. As the effectiveness of immune-mediated tumour rejection is a product of both immunization and antigen, reduced efficacy of vaccinating with self tumour antigens can be offset by using increasingly potent vaccination protocols that can activate and expand the remaining low-avidity T cells, and/or by vaccinating with a mixture of tumour antigens.

The search for broadly expressed (universal) tumour antigens has been intensified with the identification of telomerase reverse transcriptase (TERT) — the protein component of telomerase — as a potential antigenic target that is expressed in most patients with cancer. Survivin and OFA62 (the precursor for the mature form of laminin receptor) are other examples. TERT, survivin and OFA are also representatives of antigens with functions that might be essential for the maintenance of the oncogenic phenotype of the tumour cells — which has led some researchers to think that immune evasion would be reduced.

This is doubtful, as tumour cells can circumvent immune recognition through mutations in the antigen-processing pathway.

Stimulating a potent immune response.

The potency of a vaccination protocol will be a function of the magnitude of the immune response induced, type of immunity generated and how long it will persist in the patient (FIG. 1). The goal is to channel the tumour antigens into the DC-presentation pathway — to introduce the antigens into the appropriate DC subset and to induce the DC to differentiate into a potent immunostimulatory cell.

There are two general approaches to channel antigens into the DC-presentation pathway — the in vivo route, and the ex vivo route. The in vivo route is the age-old approach to vaccination, which consists of injecting antigen mixed with adjuvant into a patient, used long before it was known that a primary function of adjuvants was to mobilize the DC system. The in vivo approach is arguably the simpler…

Minimizing the risk of autoimmunity.

Cancer-vaccination protocols will invariably lead to at least some stimulation of immunity against normal gene products and, therefore, carry a risk of inducing autoimmune pathology. The underlying premise of cancer vaccination is that there must be a window of opportunity between what it takes to induce a therapeutically beneficial antitumour effect and unacceptable levels of autoimmune pathology. The peripheral immune system is populated by a spectrum of autoreactive T cells that can be divided into two general categories: low-avidity T cells that escaped central and peripheral tolerance and low- to high-avidity T cells corresponding to selected tissue-specific products that were 'ignored' by central and peripheral tolerogenic mechanisms. Depending on the effectiveness and intensity of the immunization protocol, all classes of autoreactive T cells can be activated and cause harm.

We have examined autoimmunity and vaccines previously. This is always a concer, albeit in a small fraction, but efficacy vs adverse reactions is a continuing issue. We have seen this in the COVID vaccine usage.

In reality, however, the high-avidity tissue-specific autoreactive T cells will pose the main danger.

It is important to appreciate that, from a standpoint of inducing autoimmune pathology, the circumstances prevailing during pathogen infection differ from the circumstances prevailing during antitumour vaccination with self-antigens.

Preventing immune evasion.

The genetic instability of tumour cells is well documented and so is the propensity of tumour cells to evade the immune system, through a host of genetic and epigenetic means. Tumour cells often induce the expression (B7H1, STAT1) or secretion (TGF-β, IL-10) of factors that suppress or attenuate the antitumour immune response. Conceivably, increasingly potent immunization protocols could offset the impact of such immunosuppressive factors. More troublesome are genetic changes, such as mutations in tumour antigens that make the tumour cells less susceptible to immune recognition — resulting in immune escape. Recent clinical experience indicates that this is not an idle threat.

Evasion as well as exhaustion is a major concern. Again the COVID experience, albeit a single stranded RNA virus, shows the effect of mutations.

However, immunization against several antigenic targets or against antigens that are required for maintaining the oncogenic phenotype — that is, TERT, survivin and OFA — should be able to overcome these type of mutations. The real problem is mutations in the antigen processing pathway, such as those in β2-microglobulin, TAP or components of the proteasome17,18. In this case, a single (or two) mutational events are sufficient to confer resistance to the CD8+ T-cell arm of the immune system that no immunization protocol can overcome.

In a recent 2023 Nature Medicine Editorial they note:

In February 2023, the US Food and Drug Administration awarded breakthrough designation to ***the combination of a personalized mRNA vaccine (mRNA-4157/V940) and a monoclonal antibody to the immunoinhibitory receptor PD-1 (pembrolizumab) for the treatment of patients with resected stage III/IV melanoma at high risk of recurrence****, on the basis of the unpublished (at the time of this writing) results of the randomized phase 2b KEYNOTE-942 trial.*

Patients treated with the combination of pembrolizumab and an individualized mRNA vaccine encoding up to 34 tumor-specific, mutant antigens (neoantigens) had a 44% higher rate of recurrence-free survival than that of patients treated with pembrolizumab alone, a first for an mRNA vaccine against melanoma*. Initiation of a larger phase 3 trial in patients with melanoma is now anticipated.*

In May, Rojas et al. reported results from a ***phase 1 trial in which patients with resected pancreatic cancer received chemotherapy, a monoclonal antibody to the PD-1 ligand PD-L1 (atezolizumab) and a personalized mRNA vaccine****. After a median of 18 months of follow-up, half of the vaccine recipients, all of whom had expanded neoantigen-specific T cells after vaccination,* ***remained cancer free****. For a cancer with some of the highest post-resection recurrence and mortality rates and an immunosuppressive tumor microenvironment that has frustrated immune-targeted interventions, these findings offer exciting hope of an attainable clinical advance.*

In both trials, ***neoantigens were predicted from sequenced tumor tissue****, and a customized vaccine was delivered to each patient after surgical removal of the tumor. Whether more-profound clinical responses might be achieved if vaccines were administered prior to surgery when tumor burden is high, as has been seen for the treatment of advanced melanoma with pembrolizumab in the neoadjuvant setting, or whether a neoadjuvant vaccine regimen coupled with immune checkpoint blockade might induce excessive toxicity, remains to be determined. An ongoing trial of mRNA vaccines in patients with incurable cancers may shed some light on these issues*

The above is the status clinically after some twenty years. Understanding of the immune system, technology to produce the therapeutics, and the willingness to use multiple therapeutics has allowed for a potential explosive period for vaccines. However, as has always been the case, when new techniques are deployed in a large scale there is always the concern of unintended consequences[1].

1.3 What is a Vaccine

Let us return to the fundamental question. Namely, what is a vaccine? We will spend some time examining the various approaches. Fundamentally a vaccine is any therapeutic which primes the immune system to perform the act of pathogen remediation and elimination. This can be accomplished in a pre or post infective situation. In the pre-infective situation, the immune system is primed to look out for and eliminate certain pathogens using the elements of the

[1] See NEJM https://www.nejm.org/doi/full/10.1056/NEJMp2400209?query=featured_home

immune system. Thus, polio vaccines are a prototypical example. The vaccines for preventing cervical cancer is also one. In contrast post-infective vaccines seek out targets on the pathogen after an infection, and in almost all cases this consists of infected cells, and then the immune system is activated to go after those infected cells and no other.

1.4 OVERVIEW

Our approach in this Note is to examine the current literature but doing so against the background of current constructs of immunity. The problems one sees in cancer vaccines is severalfold. First is the identification of antigens. Then the uniqueness of antigens. The next is is the complexity of the tumor microenvironment, breaking through the mess protecting tumor cells.

Add to this the ongoing mutations of tumor cells plus the exhaustion of T cells used to eliminate the malignancies. It becomes a never-ending battle if even just one tumor cells survives. Thus, we consider the following.

1. A simple review of the immune system as relates to cancer targeting. Our approach is to employ the simple graphic which predominate our general understanding. However, the immune system is dynamically changing and is not just one graphic presentation after another. It is complex and interactive, a stochastic system if you will.

2. The targets, antigens, for an immune assault must be identified. This is a significant challenge. It demands two key steps. First a cell-by-cell profiling of antigens, if possible, and second identifying the controlling or stem cell profile.

3. The tumor micro-environment, TME, is the protective amalgam of immune and other cells and proteins etc which surround, enhance, protect and nurture the malignant cells. We must understand this complex environment and must be able to deal with it.

4. We then examine vaccine options. There has been a sea state change after COVID with the introduction of mRNA vaccines. These may be useful for simple well-defined pathogens like COVID but may be complex with cancer cells.

5. The use of adjuvants has been utilized for about 100 years. Initially aluminium salts and they have evolved considerably. We briefly summarize current understanding.

6. We examine some recent results in several cancers.

2 IMMUNE SYSTEM DYNAMICS

We examine briefly some key elements of the immune system response and relate it to cancer vaccines. Basically, the paradigm is that if a cancer cell has some unique surface marker, protein, the one should be able to create a vaccine that activates the immune system to attack and eliminate the cancer cells. A simple idea, but often futile.

The following overview is one which classically one sees. It assumes that these cancer cells are in isolation and that equally the immune system cells are likewise. We see limited surface markers in cancer cells and clear attachments to immune cells. In reality these simple graphics, cartoons if you will, are quite limited in reality. As we shall note later:

1. Cancer cell targets for immune attack may be present on many cells not just cancer. In addition, these targets cover the surface of the cell along with other receptors and ligands. In a sense the cancer cell, like many others, looks like a furry ball with surface proteins everywhere. The simplistic ideas we present have serious limitations.

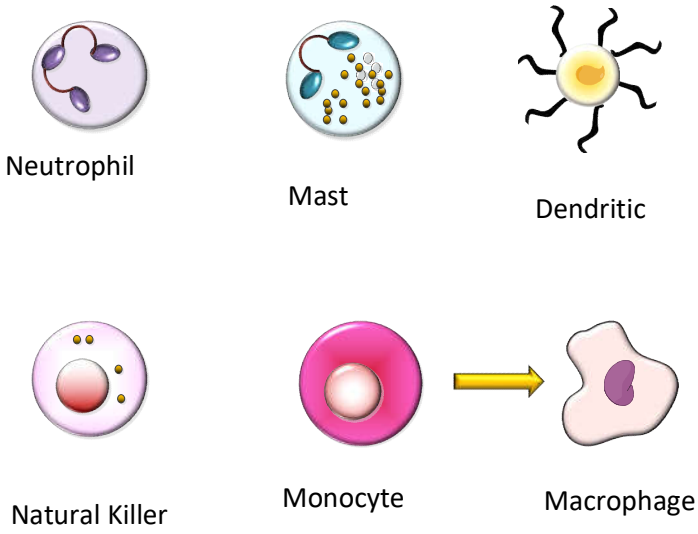
2. Cancer cells have inhibitors. PD-1 etc are just a few. Cancer cells act dramatically different from normal cells, providing for self-preservation.

3. The microenvironment of a cancer cell complex is both a protective and nurturing environment. It allows for proliferation and protection.

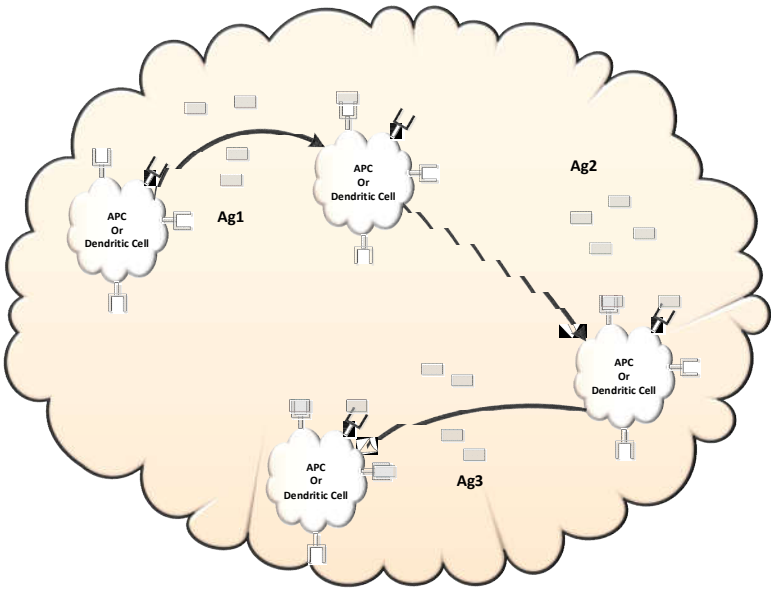
We shall examine some of these limitations as we proceed. However, the basic principles we provide an overview here apply with limitations.

2.1 INNATE

The innate system is comprised of a variety of cells and processes. The principle innate cells are depicted below. The dendritic cells, DC, and the macrophages, MΦ, are the principal ones we deal with as antigen presenting cells, APC.



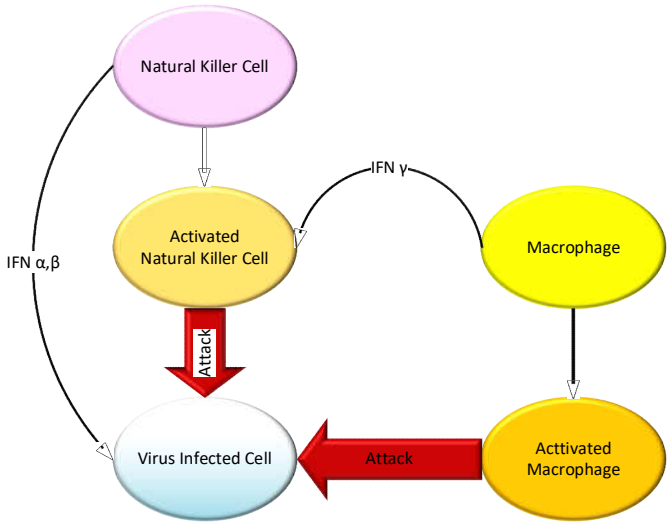
The APC roam about collecting antigens, Ag, and in turn present them to various immune system cells whose function it is to eliminate the cells that produce the Ag. This paradigm we shall use in examining cancer vaccines. Needless to say, there is much more complexity involved and we shall not detail it here (See Abbas et al).



One arm of the elimination process is shown below with Natural Killers cell, NK. Here we show the flow of Ag and signalling and then activation of NK cells which result in an attack on a viral infected cell. Again, the same paradigm can be used for cancer vaccines. The objective is twofold:

1. Identify and activate an Ag specific to that set of cancer cells

2. Activate the immune cells to respond to those Ag



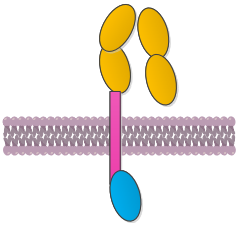
Neutrophils may also play a role comparable to NK cells.

2.2 ADAPTIVE

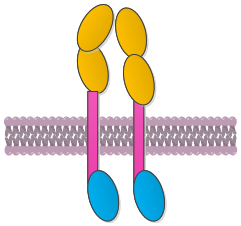
The adaptive system is composed of B and T cells. There are a large group of T cells that perform various specific tasks.

2.3 MHC

The MHC, major histocompatibility complex, 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.

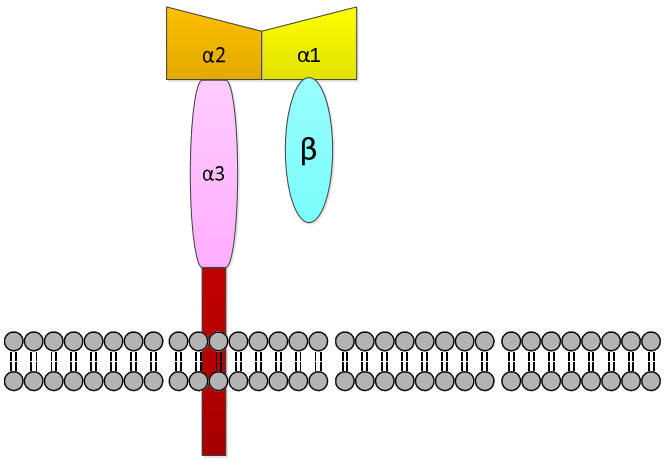


MHC I
All Cells
When something
NOT OK IN Cell

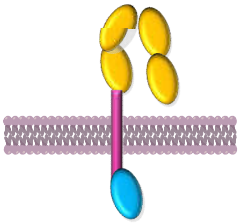


MHC II
APC Cells Only
Tells something
NOT OK Outside
Cell

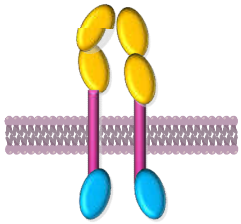
Note the difference in cell interface. MHC II appears as a dimerized molecule with two intracellular action sites. We detail the MHC I cell below with separate elements.



The two MHC proteins can be seen as below. Note the difference in structure:

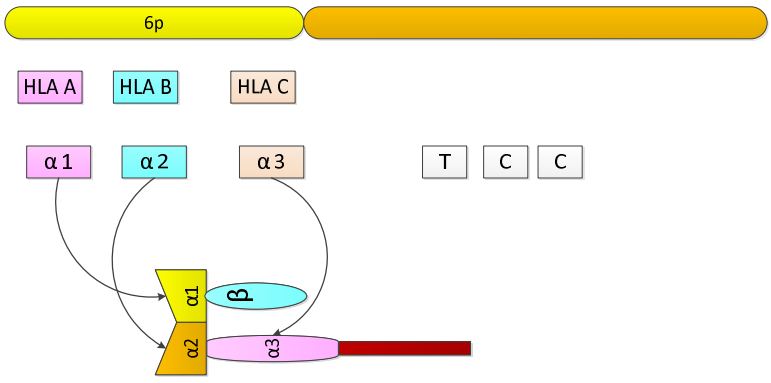


MHC I
All Cells
When something
NOT OK IN Cell

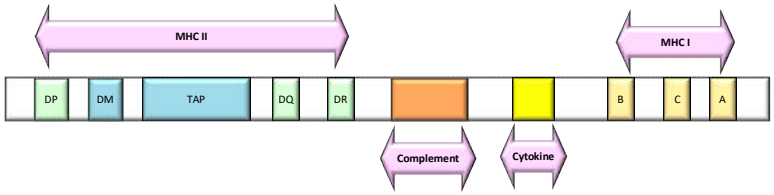


MHC II
APC Cells Only
Tells something
NOT OK Outside
Cell

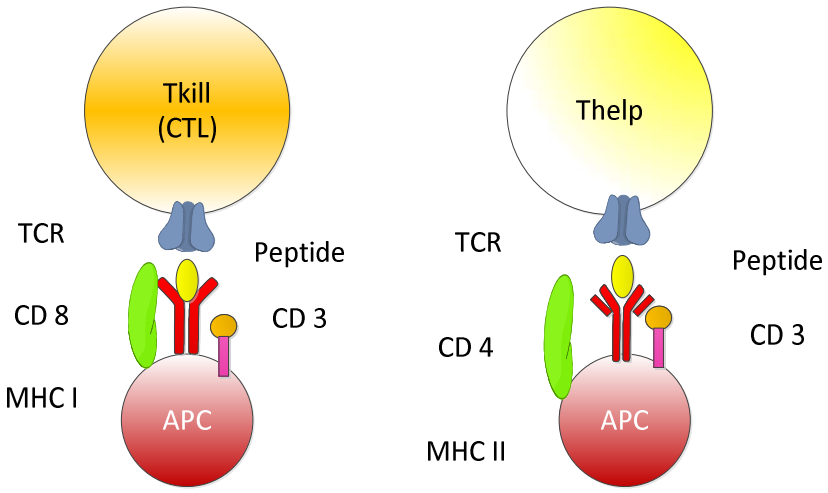
Now these are generated off of Chromosome 6 on the p region as depicted below.



More specifically the HLA contains MHC I, MHC II, cytokines and complement elements,



T_{kill} or the CTL have the ability to bind to MHC I and bind to CD-8 while the T_{help} have the ability to bind to MHC II and bind to CD-4.



2.4 T CELL PROCESS

From Abbas et al (as modified) we have the following basic principles:

1. antigen receptors of most T cells recognize only peptides displayed by major MHC on the surface of APCs. CD4+ T_{help} recognize antigens with II MHC, and CD8+ class I MHC.

2.** APCs capture protein antigens, process them, and display. Dendritic cells (DCs) are the most efficient APCs for initiating primary responses by activating T $_{naive}$ cells, and macrophages and B lymphocytes present antigens to T_{help}.* ***All nucleated cells can present class I–associated peptides, derived from cytosolic proteins, such as viral and tumor antigens, to CD8+ T cells.

3. DCs capture antigens from their sites of entry (usually through epithelia) or production (in tissues) and transport these antigens to secondary (peripheral) lymphoid organs. Naive T cells that recirculate through these organs recognize the antigens, and primary immune responses are induced in these organs.

4. Peptide antigens associated with class I MHC molecules are recognized by CD8+ T cells

5. ***The peptide-binding cleft of class I MHC molecules can accommodate peptides that are 6 to 16 amino acid residues in length****.*

6. ***Class I MHC molecules are expressed on all nucleated cells****,.*

7. ***Antigen processing is the conversion of native proteins into MHC-associated peptides****. … Thus, both extracellular and intracellular proteins are sampled by these antigen-processing*

pathways, and peptides derived from both normal self-proteins and foreign proteins are displayed by MHC molecules for surveillance by T lymphocytes.

*8. For the class **I MHC pathway, protein antigens are degraded in the proteasome, generating peptides that bind to class I MHC molecules**. Most of these antigens are synthesized in the cytosol or introduced into the cytosol from microbes or vesicles. These peptides are delivered from the cytosol to the endoplasmic reticulum (ER) by an ATP-dependent transporter called transporter associated with antigen processing (TAP). Newly synthesized class I MHC–β2-microglobulin dimers in the ER are associated with the TAP-containing peptide-loading complex and receive peptides transported into the ER. Stable complexes of class I MHC molecules with bound peptides move out of the ER, through the Golgi complex, to the cell surface.*

*9. **APCs, mainly DCs, can ingest virus-infected or tumor cells and transport their antigens into the cytosol for presentation by class I MHC molecules. This process, called cross-presentation, enables DCs to initiate CD8+ T cell responses** to the antigens of ingested cells.*

*10. pathways of MHC-restricted antigen presentation ensure that most of the body's cells are screened for the possible presence of foreign antigens. … **proteins synthesized by intracellular (cytosolic) microbes generate peptides bound to class I MHC molecules for recognition by CD8+ CTLs, which function to eliminate cells harboring intracellular infections.** The immunogenicity of foreign protein antigens depends on the ability of antigen-processing pathways to generate peptides from the proteins that bind to self MHC molecules.*

Now in the development of cancers there are many states that the T cells go through. It is worthwhile understanding these two areas since exhaustion often leads to metastasis. T cells are quite complex and present in a multiplicity of forms. As van der Leun et al note:

*While CD8+ T cells are considered major drivers of antitumour immunity, CD4+ T cells also play a prominent role in tumour control, either by promoting or inhibiting antitumour responses. **For instance, conventional CD4+ T cells (T_{conv} cells) can promote tumour control through stimulation of, among other cells, CD8+ T cells, natural killer (NK) cells and a broad range of other innate immune cell types.** In addition to this function of facilitating antitumour immune responses, Tconv cells can exert cytotoxic functions that result in killing of human leukocyte antigen (HLA) class II expressing tumour cells or inhibit tumour growth through secretion of interferon-γ (IFNγ) and tumour necrosis factor.*

In addition to the Tconv cell pool, a T follicular helper (TFH) cell-like population of CD4+ T cells that is characterized by expression of B cell lymphoma 6 (BCL-6) and the capacity to produce high levels of CXC chemokine ligand 13 (CXCL13) has been identified in multiple human tumour types77. Although the exact role of T FH cells in tumour immunity is unclear, these cells may contribute to the generation of tertiary lymphoid structures (TLS) at the tumour site and thereby shape intratumoural CD8+ T cell and B cell responses. By contrast, tumour-resident regulatory T (Treg) cells have been shown to counteract tumour-specific immune responses by suppressing the infiltration and antitumour activity of, among other cells, CD8+ T cells and macrophages.

Single-cell RNA-sequencing studies have described a variety of CD4+ T cell states, including dysfunctional CD4+ T cells, naive-like or memory CD4+ T cells, cytotoxic effector CD4+ T cells, Treg cells and TFH cells. Notably, unlike the major CD8+ T cell states, these CD4+ T cell states do not appear to be ubiquitously present in all tumour types. Another interesting observation of single-cell sequencing as well as cytometry by time of flight (CyTOF) studies has been that Treg cells in the tumour express higher levels of tumour necrosis factor receptor superfamily member 9 (TNFRSF9; encoding 4-1BB), inducible T cell costimulator (ICOS) and cytotoxic T lymphocyte-associated antigen 4 (CTLA4) than Treg cells in blood or adjacent normal tissue, possibly reflective of an activated state.

In addition, the intratumoural Treg cell pool displays substantial diversity, as shown, for example, by the variable expression levels of TNFRSF9. Furthermore, in melanoma, both T reg cells and TFH cells displayed levels of proliferation that were comparable to those observed in dysfunctional CD8+ T cells. By analogy with the dysfunctional CD8+ T cell pool, it may be hypothesized that this proliferative signature reflects a response of these cell pools to a local (antigen) signal and suggests that both Treg cells and TFH cells may play pivotal roles in the intratumoural CD4+ T cell response. ...

Recent studies using high-dimensional profiling techniques have led to an appreciation of the variety of states that are taken on by T cells in human tumours. While the nomenclature used to define these cells has varied, three major cell states — naive-like, cytotoxic and dysfunctional — have consistently been described in multiple tumour types. Notably, dysfunctional T cells do not form a homogeneous population but rather form a continuum of cell states that display increasing characteristics of dysfunction.

In addition, T cells with variable levels of dysfunction differ in functional capacity, as demonstrated by the high proliferation rate of pre-dysfunctional and in particular early dysfunctional cells and the production of CXCL13 by cells that have progressed further along the (pre)dysfunctional axis.

Antigen recognition is a — if not the — major driver of cell state diversification among tumour-infiltrating CD8+ T cells, and tumour-reactive T cells appear more prone to differentiate towards a dysfunctional state than bystander cells within the same lesions. Nevertheless, T cells with the same tumour antigen specificity can display different degrees of dysfunctionality, and the presence of tumour-reactive T cells with a low level of dysfunction may be critical for the generation of a durable antitumour response on ICB. These data are compatible with a model in which T cell dysfunctionality serves as a sensitive indicator for the presence of a tumour-reactive T cell pool, with the less dysfunctional cells within this pool being required for its renewal.

Some of the major questions that remain to be addressed are as follows: ***Which T cell states in human cancers resemble the TCF1+ T cell population that is required to maintain response to ICB in mice? What is the identity of the effector population that is ultimately responsible for tumour killing on ICB? How can we accurately identify and quantify those T cells in human cancer lesions that can both recognize surrounding tumour cells and have the capacity for long-term reinvigoration by ICB?***

Finally, the factors that drive CD8+ T cell differentiation in human tumours are presently incompletely understood, and insights into this are likely to offer new possibilities for patient stratification and therapeutic intervention.

As Gebhardt et al note:

Classical concepts of pathogen-specific T cell memory are often applied to the study of T cell responses to progressing cancers, including the analysis of tumour-infiltrating T cells (TITCs) in surgical specimens.

*Accordingly**, TITC populations are commonly classified as memory T cell subsets initially defined in infection.***

However, this approach is problematic for one simple reason: unlike bona fide memory cells that assume a relatively quiescent state following resolution of infection, a proportion of TITCs are tumour-reactive and, therefore, chronically stimulated by cancer antigens. This distinction is critically important, as unceasing antigen stimulation profoundly alters T cell differentiation trajectories, yielding TPEX cells and effector-like, tumour-reactive, exhausted CD8+ T cells (TEX cells) that are distinct from resting memory T cells.

Nevertheless, some tumour-reactive CD8+ T cells in tumour-free lymph nodes may avoid chronic antigen stimulation and align more closely with classical memory T cells. These facets of tumour-reactive T cell differentiation have far-reaching implications for the understanding of both tumour immune surveillance and cancer immunotherapy Compelling data on the relevance of TITCs to both prognosis and immunotherapy efficacy, combined with advances in the single-cell transcriptomics field, have rapidly advanced the understanding of TITC heterogeneity. A diversity of common CD8+ T cell phenotypes are associated with solid tumours, including 'naive like', 'cytotoxic', 'exhausted' or 'dysfunctional', 'resident memory' and 'effector memory' cell populations.

A major question is whether the TITCs identified in these studies recognize tumour-derived antigens, or simply accumulate due to non-specific inflammatory cues.

This issue is pertinent given that many TITCs within a tumour are irrelevant 'bystander' cells with unrelated specificities, including for pathogen-derived antigens. Several innovative studies have recently circumvented this problem by studying viral antigen-specific T cells in virally induced tumours, and by retrospective functional validation of TITC tumour specificity using TCRs identified by single-cell TCR sequencing.

This "bystander" construct is critical. T cells can accumulate, especially as we see more macrophages. Many are just incidental.

This has led to identification of markers enriched within tumour-reactive subsets of TITCs, including combinations of CD39 (encoded by ENTPD1), thymocyte selection-associated high mobility group box protein (TOX), PD1 (encoded by PDCD1), T cell immunoglobulin and mucin domain-containing protein 3 (TIM3; encoded by HAVCR2), layilin (encoded by LAYN),

granzyme B (encoded by GZMB), CD103 (encoded by ITGAE), C–X–C motif chemokine 13 (CXCL13) and C–X–C chemokine receptor type 6 (CXCR6), although as discussed below there are limitations with relying solely on these markers to identify bona fide tumour-reactive cells.

Notably, most of these markers are also enriched in CD8+ T cells with an 'exhausted' phenotype, which is consistent with this subset being enriched for tumour-reactive specificities, being more abundant within tumours compared with adjacent normal tissue and exhibiting evidence of robust clonal expansion.

Exhaustion is a common concern. One wonders if this demands continued vaccine infusions or if for each infusion and new set of antigen profiles must be obtained.

In parallel, a large body of preclinical work has demonstrated that CD8+ T cell exhaustion restrains antitumour immunity, and that direct targeting of exhausted CD8+ T cells underpins the efficacy of certain immunotherapies. Collectively, this has led the field to focus on the establishment, maintenance, heterogeneity and therapeutic manipulation of the exhausted T cell differentiation state. ...

The authors now consider the TRM cells and their impact:

The exact mechanisms through which bona fide CD8+ TRM cells or CD8+ TRM-like cells enforce local cancer surveillance are currently unclear. Moreover, whether bona fide TRM cells promote 'tumour–immune equilibrium' via direct cancer cell killing (*coupled with ongoing cancer cell division) or by secreting non-cytolytic mediators that suppress cancer cell division is unknown*[2]*.*

Secretion of tumour necrosis factor (TNF) by bona fide TRM cells is important for establishing tumour dormancy in preclinical melanoma models. Furthermore, both TNF and IFNγ produced by PD1+CD39+CD103+/– TRM-like cells are required for suppression of breast cancer micrometastases in lungs, and previously both cytokines were shown to promote tumour cell senescence.

Thus, phases of bona fide TRM cell attack on cancer cells may be interspersed by phases of relative quiescence during which co-localization may not result in bona fide TRM cell activation by dormant cancer cells.

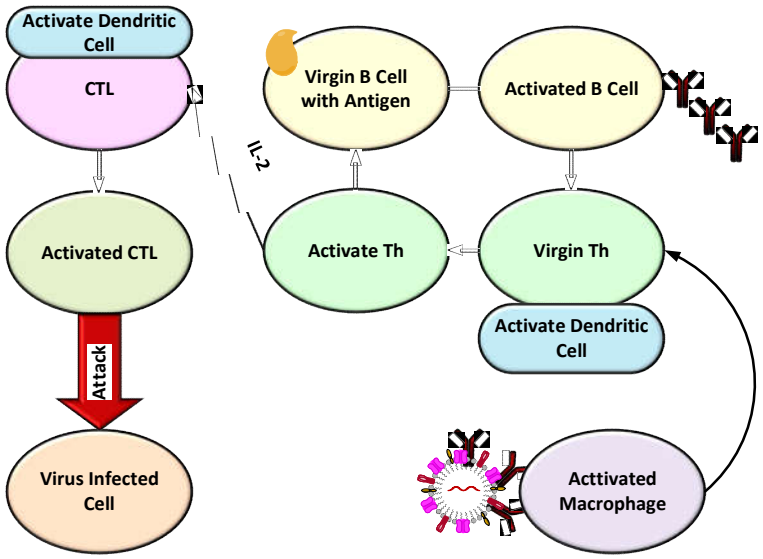
Cytokine production by bona fide TRM cells can also trigger DC maturation and epitope spreading, serving a 'sense-and-alarm' function that also recruits other immune effectors into tumours.

[2] Note from Gebhardt: *Memory T cells distribute across all manner of lymphoid and peripheral tissues. Many of the peripheral cells belong to the category of sessile, so-called* ***tissue-resident memory T cells (TRM cells)*** *that display only restricted recirculation potential. The peripheral distribution of memory T cells affords considerably broader surveillance than that afforded by their naive counterparts whose recirculation is restricted to lymph nodes. The strategic positioning of TRM cells explains their important function in tissue immunity and in the control of persisting cancer cells*

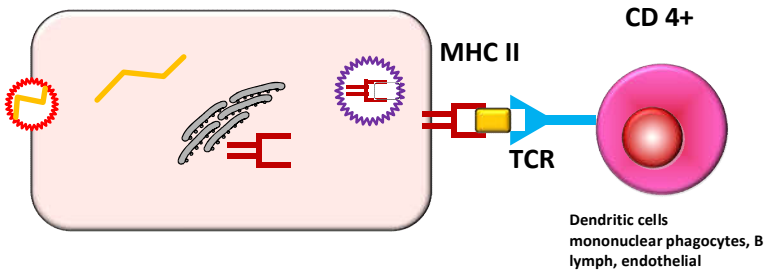
Bona fide TRM cells and TRM-like cells also highly express cytotoxic mediators such as granzyme B and perforin, and ligation of CD103 can promote autologous cancer cell killing by TITC-derived CD8+CD103+ T cell lines in vitro…

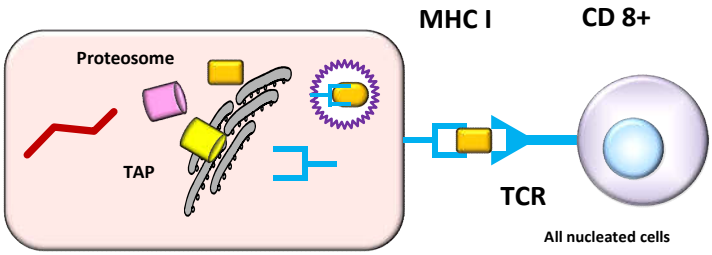
However, protection against viral infection depends stringently on TRM cell density, and bona fide TRM cell or TRM-like cell numbers in either dormant or progressively growing cancers may not reach the thresholds required for effective cancer cell killing.

The following is the classic paradigm for activating immune cells. A pathogen is caught by an APC, macrophage, which in turn with a dendritic cell activates a T cell which activates both a CTL and B cell. The activated CTL attacks the pathogen as a cell containing element whereas the B cells attack the pathogen per se. There are however multiple subtleties in this model.

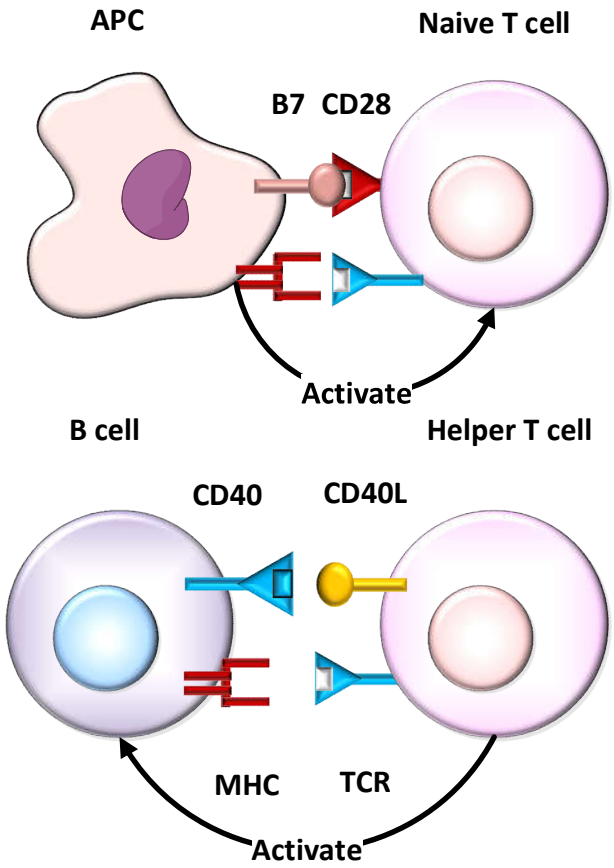


Identifying "self" is a critical issue and MHCs, or HLA in humans, are key players here. Shown below is the MHC and T cell receptor combinations. CD4 are limited to a few cell types whereas CD8 are for all nucleated cells.





Finally, the interaction between APC and T and B cells is not just via MHCs but there are other linkages required to effect the properties of the interaction. We shall see this in the case of immunotherapy.



2.5 NK T CELLS

NKT cells, natural killer T cells, are powerful cells which can mitigate pathogens. From Abbas et al:

NKT cells express markers that are characteristic of both NK cells and T lymphocytes and express αβ T cell receptors with very limited diversity. NKT cells recognize lipids and glycolipids displayed by the class I MHC–like molecule called CD1. There are several CD1 proteins expressed in humans and mice. Although their intracellular traffic pathways differ in subtle ways, all CD1 molecules bind and display lipids by a unique mechanism.

Newly synthesized CD1 molecules pick up cellular lipids and carry these to the cell surface. From here, the CD1-lipid complexes are internalized into endosomes or lysosomes, where lipids that have been ingested from the external environment are captured and new CD1-lipid complexes are then formed, which are returned to the cell surface.

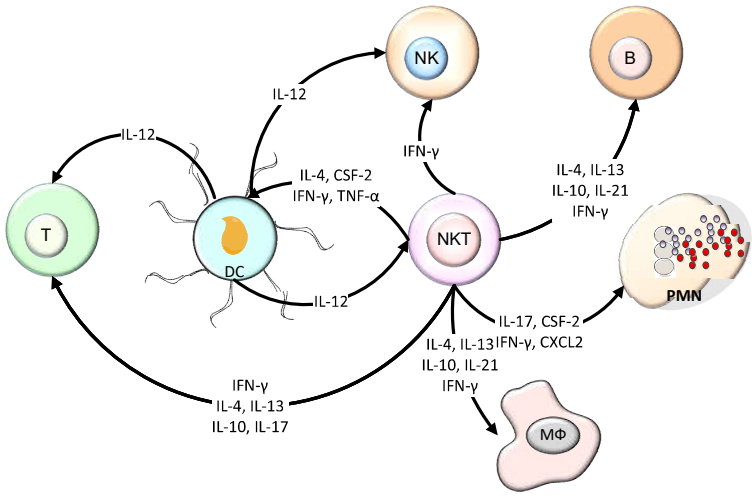
Thus, CD1 molecules acquire endocytosed lipid antigens during recycling and present these antigens without apparent processing. The NKT cells that recognize the lipid antigens may play a role in defense against microbes, especially mycobacteria (which are rich in lipid components)

…. in addition to CD4+ and CD8+ T cells, there are smaller populations of T cells that have distinct features and may serve specialized functions in host defense. The best defined of these lymphocytes are γδ T cells, natural killer T (NKT) cells, and mucosa-associated invariant T (MAIT) cells. All three of these cell types have common characteristics that distinguish them from CD4+ and CD8+ T cells. They recognize a limited number but a wide variety of antigens, many of which are not peptides, and these antigens are not displayed by class I and class II MHC molecules on APCs.

The antigen receptors of γδ T cells, NKT cells, and MAIT cells have limited diversity, suggesting that all three cell types may have evolved to recognize a small group of microbial antigens. It is also possible that these cells mainly respond not to particular antigens but to cytokines produced at sites of infection and tissue damage.

Because of these features, these T cell populations are often said to be at the crossroads of innate and adaptive immunity. All three cell types are abundant in epithelial tissues, such as the gastrointestinal tract.

We demonstrate some of these linkage below:



2.6 NK CELLS

Natural killer cells, NK, are elements of the innate immune system[3]. They often are the first cells on the task of attacking aberrant cells. Natural killer (NK) cells are a subset of bone marrow–derived lymphocytes. The NK cells are totally distinct from B or T cells. The NK cells function in **innate immune system** and they respond to kill microbe-infected cells by direct lytic mechanisms and by secreting IFN-γ. NK cells do not express clonally distributed antigen receptors like Ig receptors or T Cells Receptors and their activation is regulated by a combination of cell surface stimulatory and inhibitory receptors, the latter recognizing self MHC molecules[4]. Based on the work of Lorenzo-Herrero et al:

Natural Killer (NK) cells are cytotoxic immune cells with an innate capacity for eliminating transformed cells in a non-major histocompatibility complex (MHC) and non-tumor antigen-restricted manner.

The activation of NK cells depends on a balance of signals provided by inhibitory and activating receptors that detect changes in the patterns of expression of their ligands on the surface of tumor cells. Inhibitory NK cell receptors recognize self-proteins and transmit inhibitory signals that maintain tolerance to normal cells.

Killer cell immunoglobulin-like receptors (KIRs)[5]

[3] See Islam et al for another summary

[4] See Abbas et al

[5] From Abbas: *Killer cell Ig-like receptors (KIRs) Ig superfamily receptors expressed by NK cells that recognize different alleles of HLA-A, HLA-B, and HLA-C molecules. Some KIRs have signaling components with ITIMs in their cytoplasmic tails, and these deliver inhibitory signals to inactivate the NK cells. Some members of the KIR family have short cytoplasmic tails without ITIMs but associate with other ITAM-containing polypeptides and function as activating receptors.*

and the heterodimer CD94-Natural Killer Group 2A (NKG2A) are inhibitory receptors that recognize self-MHC class I molecules, whereas other inhibitory receptors, such as T cell immunoreceptor with Ig and ITIM domains (TIGIT) receptor, bind to other self-molecules. Transformed cells frequently downregulate MHC class I molecules, thereby avoiding recognition by CD8+ cytotoxic T cells, but concomitantly inducing the activation of NK cells by missing self-recognition.

From Laskowski et al we have the following issues regarding NK efficacy:

Despite the successes of engineered T cell immunotherapies, the clinical benefit has been limited to a fraction of patients and a few indications, thus highlighting the need for new strategies. Leveraging innate immunity to broaden the scope of antitumour responses is an attractive option.

Within the innate immune system, NK cells are specialized immune effector cells, and are suspected to have a role in tumour immunosurveillance, as suggested by the correlation of low NK cell activity with increased cancer susceptibility and higher risk of metastasis observed in both preclinical and clinical studies. NK cells develop from CD34+ progenitor cells in the bone marrow, although it is as yet unclear whether they arise from a unique set of precursor cells or from multipotent progenitors that also give rise to T lymphocytes, B lymphocytes and myeloid cells.

Unlike T cells and NKT cells, NK cells lack expression of the clonotypic TCR and the associated CD3 complex responsible for signal transduction. NK cells are generally classified under a dichotomous distribution based on the relative expression of surface proteins CD56 and CD16: CD56brightCD16low/– (immunomodulatory, cytokine-producing) and CD56dimCD16+ (cytotoxic).

Recent advancements in high-parameter cytometry and single-cell proteo-genomics, however, have led to the understanding that NK cells may, in fact, exhibit greater phenotypic heterogeneity that extends beyond these two subsets, giving rise to diverse cell populations endowed with varying functional properties. NK cells possess strong cytotoxicity and, upon forming immunological synapses with targets, elicit a potent response through the release of cytolytic granules and cytotoxic cytokines.

Moreover, they can recognize antibody-coated cells through their FcγRIIIA (CD16) receptor and trigger antibody-dependent cellular cytotoxicity (ADCC) and cytokine production.

NK cells have also been described as 'immune-regulatory' because of their ability to produce an array of cytokines and chemokines, through which they help shape B cell and T cell responses, and impact the function of dendritic cells, macrophages and neutrophils.

This broad range of attributes reveals the sophisticated network of biological mechanisms associated with NK cell function and supports the value of NK cells for immunotherapy. Memory-like function in NK cells. Early studies reported memory-like responses by NK cells in mouse models of cytomegalovirus infection, a behaviour not typically associated with innate

immune cells. In these studies, mouse NK cells, when stimulated with a combination of IL-12 and IL-18, acquired a functional phenotype characterized by increased production of IFNγ.

Interestingly, after a resting phase, these cells were able to reactivate upon cytokine stimulation or engagement of activating receptors and exhibited an enhanced IFNγ response resembling the memory-like properties of adaptive immune cells. Later, Todd Fehniger's group hypothesized that human NK cells should, likewise, be endowed with memory-like properties.

Consistent with this hypothesis, their study demonstrated that human NK cells, preactivated with IL-12, IL-15 and IL-18, followed by 1–3 weeks rest, were able to generate a robust response driven by enhanced IFNγ production upon subsequent exposure to cytokines or to K562 leukaemia cells28. Since then, many more groups have described similar memory-like function in various immunological settings, including observations of such responses in humans. ...

Because allogeneic NK cells do not cause GvHD, current NK cell therapy programmes rely largely on allogeneic sources to avoid the incumbrances associated with autologous approaches.

We have noted previously that NK cells have certain advantages and graft vs host disease is one of them. Allogenic supplies can be readily made available and such things are CAR-NK cells have shown efficacy plus no GVHD.

There are various sources from which NK cells can be derived, namely peripheral blood mononuclear cells, cord blood, immortalized cell lines, haematopoietic stem and progenitor cells (HSPCs) and induced pluripotent stem cells (iPSCs). All sources can provide clinically meaningful cell doses, are amenable to CAR receptor engineering and have transitioned into in-human studies. They, nevertheless, come with unique advantages and challenges, and may possess different underlying transcriptional, phenotypic and functional properties. NK-92, the first NK cell-based immunotherapy to receive Investigational New Drug approval by the US Food and Drug Administration (FDA) for clinical testing, is a homogeneous, immortalized NK lymphoma cell line that can be expanded ex vivo to achieve large cell numbers.

NK-92 cells lack expression of most KIRs and are thus less likely to become inhibited, which makes them attractive for cell therapy use.

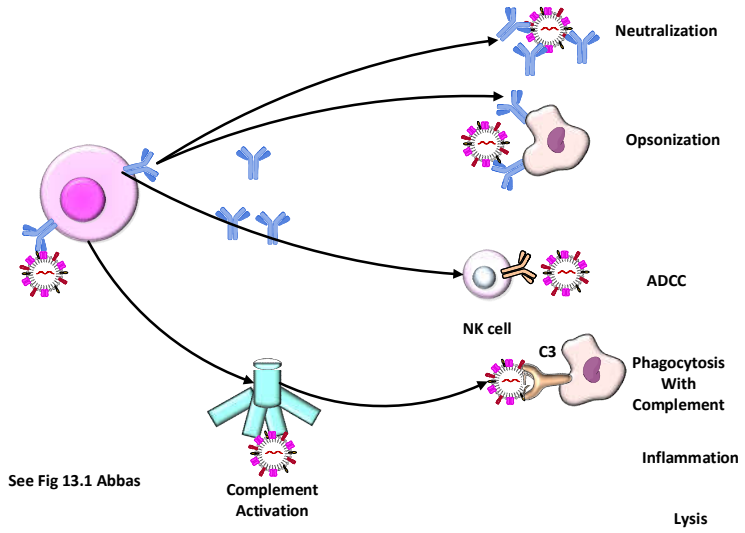
As Raskov et al note :

Human NK cells are phenotypically characterized by the expression of neural cell adhesion molecule (NCAM/CD56) and the absence of CD3 and T cell receptors. They are mainly present in the lymph nodes and peripheral circulation where they constitute approximately 2 % of the leukocytes in the blood. The lifespan of human NK cells is approximately two weeks with an estimated doubling time in vivo of approximately two weeks. If stimulated continuously, peripheral blood NK cells can achieve up to 30 population doublings before entering senescence.

In the defense against cancer, NK cells participate particularly in the immune surveillance of circulating tumor cells. Entering the circulation, the majority of tumor cells are eliminated by NK cells within 24 h; however, if and when the cancer advances, the activity of circulating NK cells generally decreases with disease progression.

The activation of NK cells occur in a matter of hours, whereas naïve T cells activate and differentiate into effector T cells over the course of 1–2 weeks. Activating and inhibitory NK-cell receptors scan the surface of the potential target cells for damage-associated molecular patterns (DAMPs) and pathogen-associated patterns (PAMPs), which represent intracellular changes such as genetic damage and cellular stress. A multitude of receptor signals control the activation; the NK cell will only activate if the activation receptors are not overruled by the inhibitory receptors. In addition, the NK receptors detect changes in healthy self-proteins (e.g. lack/loss or abnormalities in MHC-1) on the target cells, and if found, the NK cells immediately kill the target by releasing lytic toxins into the immune synapse without the need for prior antigen sensitization.

See graphically below a comparison:



They continue:

The two major groups of NK receptors are the inhibitory killer-cell immunoglobulin-like receptors (KIRs) and the activating natural cytotoxicity receptors (NCRs).

NK cells use dual mechanisms to kill their target: the direct killing process (natural cytotoxicity) and the antibody-dependent cell-mediated cytotoxicity (ADCC). In the former, the NK cell recognizes the activating ligands on the target cell surface.

In ADCC, the NK cell receptor CD16 (FCγRIIIA) ligate the Fc portion of IgG antibodies bound to antigenic molecules on target cell surfaces. Both mechanisms are initiated by the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domain of activating receptors.

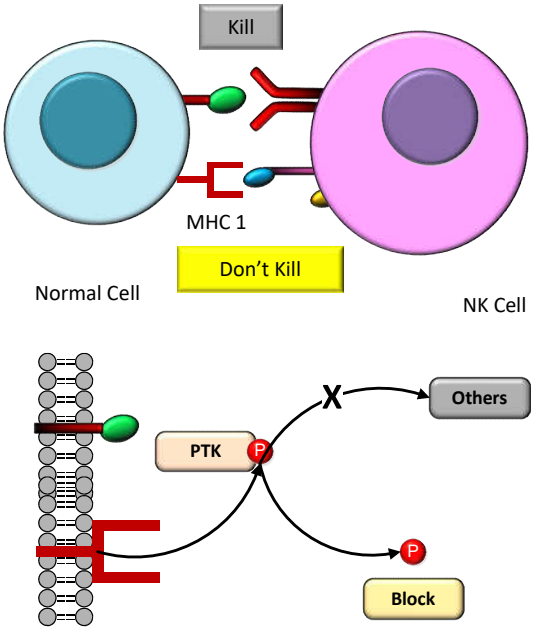
The following exocytosis of lytic granules results in immediate release of toxins (granzymes, granulysin, and perforin) into the immune synapse and death receptor ligands, e.g. tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) engaging death-receptors on the target cell. The ADCC process cleaves the CD16 molecules from the NK cell surface and the recovery of CD16 takes several days. The shedding of the extracellular CD16 domain is caused by the proteolytic cleavage by disintegrins and metalloproteinases, and it may be an important factor in NK cell detachment from the target cell after the killing.

Although the ADCC process is temporarily impaired due to slow recovery, NK cells are still capable of serially killing multiple targets (>30 cells) through natural cytotoxicity before entering senescence. An approach to optimize the efficacy of immunotherapy is to improve the recruitment of cytotoxic immune effector cells to the tumors and facilitate ADCC by the use of co-stimulatory signals from bispecific and trispecific antibodies. These synthetic antibodies bind to both tumor cells and effector cell, such as CD64 or CD16 on effector cells and CD30 on target cells (e.g. lymphoma cells).

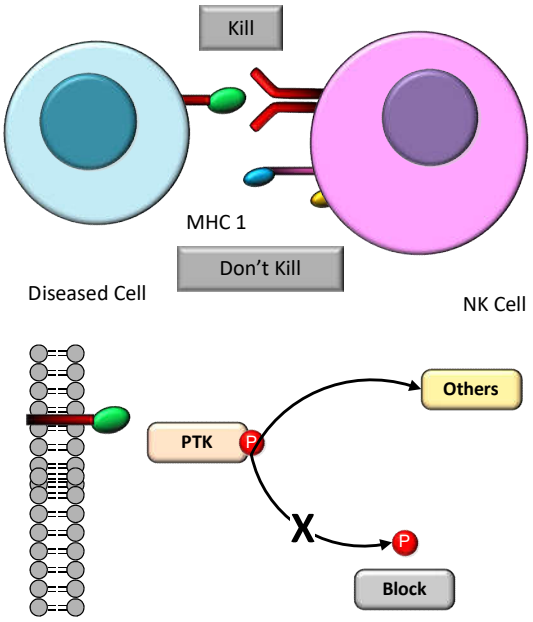
Ligand binding of CD16 on NK cells facilitates ADCC and lysis of target cells. For example, AFM13 is a novel NK cell-recruiting antibody that targets CD16A and CD30 and may provide a new treatment option for patients with relapsed or refractory Hodgkin lymphoma

The basic principles of NK activation is shown below:

First the NK cell is a useful cell for targeting. It has less complexity than a T cell and responds quickly. The typical response mechanism is shown below. First if the NK see a cell with an Ag but also an MHC 1 on the surface the action to release cytokines is inhibited. The simple construct is shown below.

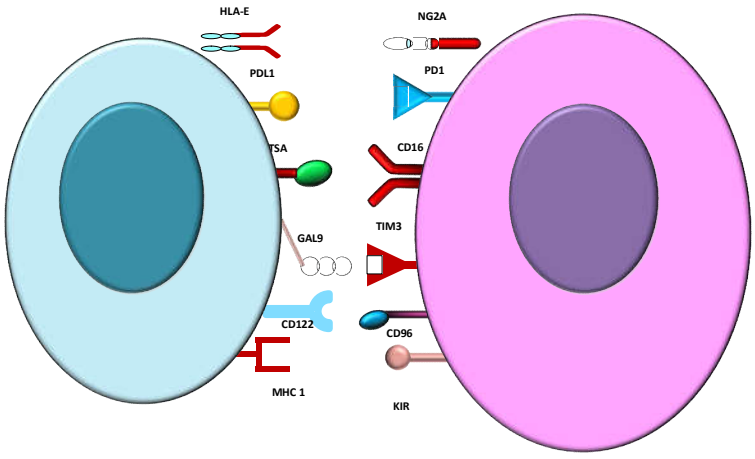


If, however the Ag does not have an MHC 1 on the surface as below then we have a release and the cell is attacked. This we show below.



Now NK cells have a large number of surface ligands and receptors. We show a typical example based on Carotta below:

See Carotta



Key to many of these are the KIR receptors. The KIR receptors will play an important role as we discuss latter. The authors continue:

Killer cell Ig-like receptors (KIRs) Ig superfamily receptors expressed by NK cells that recognize different alleles of HLA-A, HLA-B, and HLA-C molecules.

Some KIRs have signaling components with ITIMs in their cytoplasmic tails, ***and these deliver inhibitory signals to inactivate the NK cells.*** *Some members of the KIR family have short cytoplasmic tails without ITIMs but associate with other ITAM-containing polypeptides and function as activating receptors.*

Activating receptors, including, but not limited to, killer cell lectin-like receptor K1 (KLRK1—best known as NKG2D), DNAX accessory molecule-1 (CD226—best known as DNAM-1) and the natural cytotoxicity receptors NKp46, NKp44, and NKp30, recognize stress-inducible ligands on tumor cells that are scarcely expressed in their normal counterparts. Natural killer group 2D (NKG2D) is a particularly relevant activating receptor, which recognizes a group of stress-inducible molecules termed MHC class I polypeptide-related sequence A and B (MICA and MICB) and UL16 binding protein molecules (ULBP1-6), which are restrictedly expressed on stressed and transformed cells.

Thus, by this complex pattern of receptors, NK cells may kill a broad range of cancer cells. Indeed, the engagement of activating receptors by tumor-expressed ligands, along with a lack of co-engagement of an appropriate number of inhibitory receptors, results in the exocytosis of cytotoxic granules containing perforin and granzymes that induce apoptotic cell death of the target cells.

NK cells have a strong potential for cancer attack. The concern is that when they do attack they do so in a rather ruthless manner, but effectively. As part of the innate immune system their response once activated is immediate.

Additionally, NK cells can eliminate target cells through Fas ligand and tumor necrosis factor (TNF)-related apoptosis-inducing signals. Finally, NK cells may also kill tumor cells bound by specific IgG antibodies through Fc RIII receptors (also named as CD16s), a process known as antibody-dependent cellular cytotoxicity (ADCC).

The latter is a relevant process underlying the therapeutic activity of certain monoclonal antibodies. NK cells also regulate the innate and adaptive immune response through the secretion of cytokines with potent antitumor activity, such as interferon-gamma (IFN-γ).

As Bassani et al have recently noted regarding the TME and the NK cells:

Immune cells, as a consequence of their plasticity, can acquire altered phenotype/functions within the tumor microenvironment (TME). Some of these aberrant functions include attenuation of targeting and killing of tumor cells, tolerogenic/immunosuppressive behavior and acquisition of pro-angiogenic activities. Natural killer (NK) cells are effector lymphocytes involved in tumor immunosurveillance. In solid malignancies, tumor-associated NK cells (TANK cells) in peripheral blood and tumor-infiltrating NK (TINK) cells show altered phenotypes and are characterized by either anergy or reduced cytotoxicity.

Here, we aim at discussing how NK cells can support tumor progression and how induction of angiogenesis, due to TME stimuli, can be a relevant part on the NK cell-associated tumor supporting activities.

We will review and discuss the contribution of the TME in shaping NK cell response favoring cancer progression. We will focus on TME-derived set of factors such as TGF-β, soluble HLA-G, prostaglandin E2, adenosine, extracellular vesicles, and miRNAs, which can exhibit a dual function.

This rather strange action of the NK cells is also a feature in macrophages as well. The TME seems to be a fertile ground for not only cancer cell growth but the adoption of what would be cancer killing cells as supportive ones instead. Whether this becomes another set of targets has been considered by others and we believe that it has substantial merit. They continue:

On one hand, these factors can suppress NK cell-mediated activities but, on the other hand, they can induce a pro-angiogenic polarization in NK cells. Also, we will analyze the impact on cancer progression of the interaction of NK cells with several TME-associated cells, including macrophages, neutrophils, mast cells, cancer-associated fibroblasts, and endothelial cells. Then, we will discuss the most relevant therapeutic approaches aimed at potentiating/restoring NK cell activities against tumors.

Finally, supported by the literature revision and our new findings on NK cell pro-angiogenic activities, we uphold NK cells to a key host cellular paradigm in controlling tumor progression and angiogenesis; thus, we should bear in mind NK cells like a TME-associated target for anti-tumor therapeutic approaches.

As Lopez-Soto notes:

NK cells can exert robust antimetastatic functions independent of MHC-mediated antigen presentation via at least three pathways:

1. ***the release of PRF1- and GZMB-containing pre-formed granules;***

2. ***the secretion of IFNG; and***

3. ***the exposure of death receptor ligands, including FASLG and TRAIL.***

Thus, at odds with T lymphocytes (which require priming from antigen-presenting cells) NK cells are continuously poised to kill damaged, infected, or (pre)malignant cells. Such a potent cytotoxic activity is mainly regulated by the interplay between inhibitory and activatory signals originating at the plasma membrane of NK cells from NKIRs and NKARs, respectively. NKIRs keep the effector functions of NK cells at bay upon interaction with ligands expressed by normal and healthy cells.

Conversely, NKARs promote the effector functions of NK cells as they recognize a wide panel of ligands that are specifically upregulated in response to potentially detrimental perturbations of homeostasis, including DNA damage and viral infection.

NKIRs and NKARs virtually operate as mutual antagonists as they contain intracellular domains that inhibit or activate the phosphorylation-dependent signal transduction cascade leading to NK cell activation…

In vitro, NK cells have been shown to kill cancer cell lines of different histological origin, virtually irrespective of derivation (primary tumors versus metastatic lesions), including malignant cells with stem-like features.

The stem like features is an important observation. They are the cells, if they exist, that are critical to eliminate.

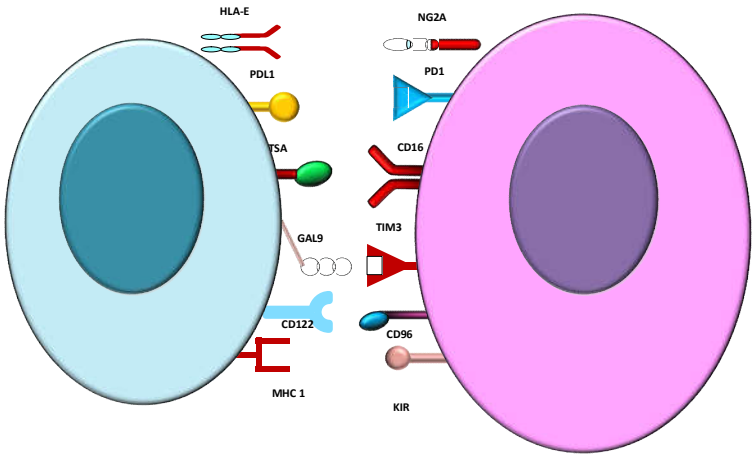
Accordingly, Klrk1/ mice develop transgene-driven lymphomas and prostate carcinomas at increased incidence compared with WT mice. Moreover, transgene-driven overexpression of NKG2D ligands renders multiple murine cancer cells that normally form tumors upon inoculation into immunocompetent syngeneic hosts sensitive to rejection.

Moreover, selective depletion experiments demonstrated a role for NK cells in the control of methylcholanthrene-driven fibrosarcoma. However, Klrk1/ mice are equally sensitive to methylcholanthrene- driven carcinogenesis as their WT counterparts and develop diethylnitrosamine-induced hepatocellular carcinomas at a comparatively increased incidence. Furthermore, Tlr3/ mice, which are characterized by NK cell hyporesponsiveness, are more sensitive to metastatic spread than WT mice, yet do not differ from WT mice in terms of spontaneous carcinogenesis (nor in terms of primary growth of subcutaneously inoculated murine melanoma, breast carcinoma, or colorectal carcinoma cells).

Finally, NK cells generally represent a minor fraction of the immunological infiltrate of most established solid tumors in humans and have limited prognostic value compared with other tumorinfiltrating lymphocytes such as CD8+ CTLs or CD4+CD25+FOXP3+ TREG cells

We seem to understand that albeit NK presence but the most facilitating cells may be the macrophages. Likewise, the interaction between the NK cell and the PCa cell results from interactions as shown below.

See Carotta



2.7 MACROPHAGES

Macrophages play a critical positive and negative role in cancer proliferation We know the typical paradigm of macrophages is shown below. Namely the macrophage roams around various cells and picks up "stuff" some of the stuff is an Ag. It the presents the Ag to T cells and the process begins with both T and B immune reactions. As Abbas et al describe them:

Phagocytes, including neutrophils and macrophages*, are cells whose primary function is to ingest and destroy microbes and remove damaged tissues. The functional responses of phagocytes in host defense consist of sequential steps: recruitment of the cells to the sites of infection, recognition of and activation by microbes, ingestion of the microbes by the process of phagocytosis, and destruction of ingested microbes.*

In addition, through direct contact and by secreting cytokines, phagocytes communicate with other cells in ways that promote or regulate immune responses. Blood neutrophils and monocytes are both produced in the bone marrow, circulate in the blood, and are recruited to sites of inflammation. Although both are actively phagocytic, they differ in significant ways.

(i) The neutrophil response is more rapid and the lifespan of these cells is short, whereas monocytes become macrophages in the tissues, can live for long periods, and so

(ii) the macrophage response may last for a prolonged time. Neutrophils mainly use cytoskeletal rearrangements and enzyme assembly to mount rapid, transient responses, whereas macrophages rely mostly on new gene transcription.

A major function of macrophages in host defense is to ingest microbes by the process of phagocytosis and then to kill the ingested microbes. The mechanisms of phagocytosis and killing, … include formation of cytoplasmic membrane–bound organelles that contain the microbes, the fusion of these organelles with lysosomes, the enzymatic generation of reactive oxygen and nitrogen species in the lysosome that are toxic to microbes, and digestion of microbial proteins by proteolytic enzymes. In addition to ingesting microbes, macrophages ingest necrotic host cells, including cells that die in tissues because of the effects of toxins, trauma or interrupted blood supply, and neutrophils that die after accumulating at sites of infection. This is part of the cleaning up process after infection or sterile tissue injury.

Macrophages also recognize and engulf apoptotic cells before the dead cells can release their contents and induce inflammatory responses. Throughout the body and throughout the life of an individual, unwanted cells die by apoptosis as part of many physiologic processes, such as development, growth, and renewal of healthy tissues, and the dead cells are eliminated by macrophages. Macrophages are activated by microbial substances to secrete several different cytokines that act on endothelial cells lining blood vessels to enhance the recruitment of more monocytes and other leukocytes from the blood into sites of infections, thereby amplifying the protective response against the microbes. Other cytokines act on leukocytes and stimulate their migration to tissue sites of infection or damage.

Macrophages serve as antigen-presenting cells (APCs) that display fragments of protein antigens to and activate T lymphocytes. This function is important in the effector phase of T cell–mediated immune responses. Macrophages promote the repair of damaged tissues by stimulating new blood vessel growth (angiogenesis) and synthesis of collagen-rich extracellular matrix (fibrosis). These functions are mediated by cytokines secreted by the macrophages that act on various tissue cells.

Macrophages can acquire distinct functional capabilities, depending on the types of activating stimuli they are exposed to. The clearest example of this is the response of macrophages to different cytokines made by subsets of T cells.

Some of these cytokines activate macrophages to become efficient at killing microbes, called classical activation, and these cells are called M1 macrophages.

Other cytokines activate macrophages to promote tissue remodeling and repair, called alternative activation, and these cells are called M2 macrophages.

These different pathways of activation and the cytokines involved. The relationship between blood monocyte subsets, discussed earlier, and macrophage subsets is not well understood, but classical (inflammatory) monocytes and M1 macrophages share functional properties. Macrophages may also assume different morphologic forms after activation by external stimuli, such as microbes. Some develop abundant cytoplasm and are called epithelioid cells because of their resemblance to epithelial cells of the skin. Activated macrophages can fuse to form multinucleated giant cells, which occurs frequently in certain types of microbial infections, such as with mycobacteria, and in response to indigestible foreign bodies.

From Salinas et al further delineate the M1 and M2 distinctions:

Macrophages can be divided schematically into two main classes in line with the Th1/Th2 dichotomy.

1. M1 macrophages (classically activated cells) originate upon encounter with IFN[6]-and microbial stimuli such as LPS and are characterized by IL-12high and IL-23 production and consequent activation of polarized type I T cell response, cytotoxic activity against phagocytozed microorganisms and neoplastic cells, expression of high levels of ROI, and good capability as APCs.

In general, M1 macrophages act as soldiers: they defend the host from viral and microbial infections, fight against tumors, produce high amounts of inflammatory cytokines, and activate the immune response.

2. On the other hand, distinct types of M2 cells differentiate when monocytes are stimulated with IL-4 and IL-13 (M2a), with immune complexes/TLR ligands (M2b), or with IL-10 and glucocorticoids (M2c).

Hallmarks of M2 macrophages are IL-10 high IL-12 low IL-1ra high IL-1 decoyRhigh production, CCL17 and CCL22 secretion, high expression of mannose, scavenger and galactose-type receptors, poor antigen-presenting capability and wound-healing promotion.

Further, M2 express specific change in some metabolic pathways: arginine metabolism is oriented toward the production of ornitine and polyamine instead of citrulline and NO.

M2 cells are workers of the host: they promote scavenging of debris, angiogenesis, remodeling and repair of wounded/damaged tissues. Of note, M2 cells control the inflammatory response by down-regulating M1-mediated functions.

In addition, M2 macrophages are competent effector cells against parasitic infections . The loss of equilibrium of M1 and M2 cell number may lead to pathological events: an M1 excess could induce chronic inflammatory diseases, whereas an uncontrolled number of M2 could promote severe immune suppression.

As Quaranta and Schmid note:

*Macrophages originate from **three different developmental pathways**.*

*All tissue embryonic macrophages derive from macrophage precursors in the yolk sac and fetal liver. During adulthood, fetal macrophages are replaced gradually by macrophages derived from bone marrow **hematopoietic stem cell (HSCs)**.*

[6] From Abbas et al, *IFN-γ activates macrophages to kill phagocytosed microbes. Macrophage activation resulting in increased microbicidal activity is called classical macrophage activation, to be contrasted with an alternative activation pathway that is induced by Th2 cytokines; these types of macrophage activation are described in more detail later.*

Some types of tissue resident macrophages, including bone osteoclasts, ***epidermal Langerhans*** *cells, lung alveolar macrophages, microglia and* ***liver Kupffer*** *cells develop from* ***embryonic macrophages*** *and persist in adult tissues independently of replenishment by Ly6Chigh monocytes originated from HSCs during adulthood.*

*Instead, other types of tissue macrophages such as intestine, dermis, heart and pancreas macrophages u****ndergo a continuous turnover in adulthood by recruitment of circulating monocytes which differentiate into macrophages upon tissue infiltration****.*

Infiltrating monocytes derived from HSCs are also the main source of macrophage replenishment into inflamed and remodeling tissues, and this process is driven by cytokines and chemokines, such as C-C motif chemokine ligand (CCL) 2, CCL5 and macrophage colony stimulating factor-1 (CSF-1).

*There are different markers to identify monocyte-macrophages diversity in human and mouse. In human, c****irculating monocytes, which originate from the bone marrow, can be classified in two subsets****:*

(i) CD14+ CD16neg 'inflammatory' or 'classical' and

(ii) CD14+ CD16+ 'patrolling' or 'non-classical' monocytes.

In the same way, mouse 'inflammatory' monocytes are classified as CD11b+ Ly6Chigh CCR2high CX3CR1low, in contrast 'patrolling' monocytes are CD11b+ Ly6Glow CCR2low CX3CR1high.

Patrolling monocytes monitor the microvasculature under steady-state *conditions and rarely extravasate into tissue. However, they can rapidly accumulate in lung metastatic tissue and inhibit cancer cell seeding and growth by exerting anti-tumour functions through recruitment of NKs. Macrophages are a population of heterogeneous and plastic cells.*

Once resident in tissues, macrophages acquire a distinctive phenotype in response to different signals present in the immediate microenvironment. Environmental stimuli, like IFN or microbial products, like the lipopolysaccharide

From Ruffell and Coussens:

Macrophages produce an array of cytokines, chemokines, polypeptide growth factors, hormones, matrix-remodeling proteases, and metabolites, many of which possess tumor-promoting activities. A caveat to some of these reported activities is that many findings originate from cell culture studies utilizing neoplastic myeloid cell lines or bone marrow-derived macrophages and, therefore, cannot account for the complex milieu of polarization signals to which macrophages would be exposed in vivo. This includes the aforementioned CSF-1 and CCL2, prostaglandin E2 (PGE2), and damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 protein (HMGB1), extracellular ATP, and degraded extracellular matrix components …

Macrophages are well described regulators of tumor angiogenesis, with supporting evidence derived from both clinical and experimental studies in which much of their capability is associated with vascular endothelial growth factor (VEGF) signaling. This includes macrophage production of VEGF-A, production of VEGF homologs such as placental growth factor, enhancement of VEGF-A bioavailability through matrix metalloproteinase (MMP)-9 activity, and induction of VEGF-A production by endothelial cells via WNT7B expression. VEGF-A drives the formation of abnormal vasculature in tumors, consisting of excessive branching, dead-end vessels, and vessel leakiness, that, together, impact tumor hemodynamics and drug delivery.

VEGF antagonists induce vascular normalization, and several studies have reported increased uptake of chemotherapeutics associated with this process, likely because of reduced vessel leakiness and interstitial fluid pressure. Although macrophages are not necessarily a dominant source of VEGF-A in all tumor tissues, specific deletion of VEGF-A in macrophages via lysozyme M promoter-driven Cre recombinase revealed their role in driving abnormal vascular phenotypes in tumors.

Importantly, similar to the use of VEGF antagonists, tumors in these mice were more sensitive to chemotherapy, although, unexpectedly, they also grew at a faster rate because of improved tissue perfusion and reduced hypoxia in the absence of therapeutic intervention…

As Palma et al note:

Macrophages derived from monocyte precursors undergo specific polarization processes which are influenced by the local tissue environment: classically activated (M1) macrophages, with a pro-inflammatory activity and a role of effector cells in Th1 cellular immune responses, and alternatively activated (M2) macrophages, with anti-inflammatory functions and involved in immunosuppression and tissue repair.

At least three different subsets of M2 macrophages, namely, M2a, M2b, and M2c, are characterized in the literature based on their eliciting signals.

We shall examine the M1 and M2 latter again when considering the tumor micro environment. But we shall continue here:

The activation and polarization of macrophages is achieved through many, often intertwined, signaling pathways. To describe the logical relationships among the genes involved in macrophage polarization, we used a computational modeling methodology, namely, logical (Boolean) modeling of gene regulation.

We integrated experimental data and knowledge available in the literature to construct a logical network model for the gene regulation driving macrophage polarization to the M1, M2a, M2b, and M2c phenotypes. Using the software GINsim and BoolNet, we analyzed the network dynamics under different conditions and perturbations to understand how they affect cell polarization. Dynamic simulations of the network model, enacting the most relevant biological

conditions, showed coherence with the observed behavior of in vivo macrophages. The model could correctly reproduce the polarization toward the four main phenotypes as well as to several hybrid phenotypes, which are known to be experimentally associated to physiological and pathological conditions.

We surmise that shifts among different phenotypes in the model mimic the hypothetical continuum of macrophage polarization, with M1 and M2 being the extremes of an uninterrupted sequence of states. Furthermore, model simulations suggest that anti-inflammatory macrophages are resilient to shift back to the pro-inflammatory phenotype.

M1 and M2 are critical macrophage players.

2.8 IMMUNOTHERAPY

Cancer immunotherapy has had many advances over the past decade. However there still is a great number of new options becoming available. These options present the opportunity for targeting individual patients and their specific lesions. Many cases present as relatively indolent in nature whereas there is a small number which are highly aggressive. Understanding and identifying them is still a work in progress.

However, we see options for doing so by using PCa cell surface markers. Using them we than have a variety of immunotherapeutic approaches. We show some of these below. Namely we look at stem cells, progression cells, and the tumor micro-environment. For the cell targets we look for surface markers. Then we can use antibodies, immune cells and even viral attacks. Our recent result considers the first two.

Karp et al present the immunotherapy techniques of checkpoint blockade. They note as follows:

Many cancers camouflage themselves from the immune system by producing molecules that inhibit T-cell recognition and activity.

Multiple clinically important stimulatory checkpoint molecules are members of the TNF receptor superfamily—CD27, CD40, OX40, GITR, and CD137. CD28 and ICOS are two additional stimulatory checkpoint molecules that belong to the B7-CD28 superfamily.

Some of these blockade elements are:

1. *CD27 is vital for T-cell memory and is also a memory marker of B cells (Hendriks et al., 2000). CD27 binds to its ligand, CD70, on lymphocytes and dendritic cells. CDX-1127, an agonistic anti-CD27 monoclonal antibody, has been shown to be effective in the context of TCR stimulation.*
2. *CD28 is constitutively expressed on almost all human CD4+ T cells and on approximately half of all CD8 T cells and promotes T-cell expansion following binding with its two ligands CD80 and CD86 expressed on dendritic cells.*

3. *CD40 is found on a variety of immune cells including antigen presenting cells; its ligand is CD154, which triggers T-cell activation and differentiation.*
4. *CD122 is the IL-2Rβ subunit and is known to increase proliferation of CD8+ effector T cells.*
5. *CD137 (aka 4-1BB) is bound by CD137 ligand, resulting in T-cell proliferation. CD137-mediated signaling is also known to protect T cells and, in particular, CD8+ T cells from activation-induced cell death.*
6. *OX40, also called CD134, has CD252 as its ligand. Like CD27, OX40 promotes the expansion of effector and memory T cells; however, it is also noted for its ability to suppress the differentiation and activity of Tregs and also for its regulation of cytokine production.* ***OX40's value as a drug target primarily lies in the fact that it is only upregulated on the most recently antigen-activated T cells within inflammatory lesions. Anti-OX40 monoclonal antibodies have been shown to have clinical utility in advanced cancer. OX40 agonists in development include MEDI0562, MEDI6469, and MEDI6383.***
7. *GITR, short for glucocorticoid-induced TNFR family–related gene, prompts T-cell expansion, including Treg expansion. The ligand for GITR is mainly expressed on antigen presenting cells. Antibodies to GITR have been shown to promote an antitumor response through loss of Treg lineage stability.*
8. *ICOS, short for inducible T-cell costimulator, also called CD278, is expressed on activated T cells. Its ligand is ICOSL, expressed mainly on B cells and dendritic cells. The molecule seems to play an important role in T-cell effector function and has spawned a number of new exciting anticancer agents.*

Inhibitory checkpoint factors are listed in the following from the above authors:

1. *A2AR, short for adenosine A2A receptor, is regarded as an important checkpoint in cancer therapy because adenosine activation of the A2A receptor is a negative immune feedback loop and the tumor microenvironment has relatively high concentrations of adenosine.*
2. *B7-H3, also called CD276, is a coinhibitory, although it was originally understood to be a costimulatory molecule MGA271, and is an Fc-optimized monoclonal antibody that targets B7-H3.*
3. *B7-H4, also called VTCN1, is expressed by tumor cells and TAMs and plays a role in tumor escape.*
4. ***CTLA-4, short for CTL-associated protein 4 and also called CD152, is the target of ipilimumab (Yervoy), which gained FDA approval in March 2011. Expression of CTLA-4 on Treg cells serves to control T-cell proliferation.***
5. *IDO, short for indoleamine 2,3-dioxygenase, is a tryptophan catabolic enzyme with immune-inhibitory properties. Another important molecule is TDO, tryptophan 2,3-dioxygenase. IDO is known to suppress T and NK cells, generate and activate Tregs and MDSCs, and promote tumor angiogenesis. IDO inhibitors are under active development.*

6. *LAG3, short for lymphocyte activation gene-3, works to suppress an immune response by action to Tregs as well as direct effects on CD8+ T cells. BMS-986016 is an anti-LAG3 monoclonal antibody.*
7. ***PD-1, short for programmed death 1 receptor, has two ligands, PD-L1 and PD-L2. This checkpoint is the target of pembrolizumab and nivolumab, among others. These gained FDA approval in September 2014 and has revolutionized the treatment of multiple cancers, including lung, head and neck, and melanoma. An advantage of targeting PD-1 is that it can restore immune function in the tumor microenvironment.***
8. *TIM-3, short for T-cell immunoglobulin domain and mucin domain 3, is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. TIM-3 acts as a negative regulator of Th1/Tc1 function by triggering cell death on interaction with its ligand, galectin-9.*
9. *VISTA, short for V-domain Ig suppressor of T-cell activation, is primarily expressed on hematopoietic cells so that consistent expression of VISTA on leukocytes within tumors may allow VISTA blockade to be effective across a broad range of solid tumors.*

The most prominent of the above has been PD-1. As Alsaab et al note:

Several cancers are highly refractory to conventional chemotherapy. The survival of tumors in several cases is assisted by checkpoint immunomodulation to maintain the imbalance between immune surveillance and cancer cell proliferation.

Check point antibody inhibitors, such as anti-PD-1/PD-L1, are a novel class of inhibitors that function as a tumor suppressing factor via modulation of immune cell-tumor cell interaction. These checkpoint blockers are rapidly becoming a highly promising cancer therapeutic approach that yields remarkable antitumor responses with limited side effects. In recent times, more than four check point antibody inhibitors have been commercialized for targeting PD-1, PDL-1, and CTLA-4.

Despite the huge success and efficacy of the anti-PD therapy response, it is limited to specific types of cancers, which attributes to the insufficient and heterogeneous expression of PD-1 in the tumor microenvironment. Herein, we review the current landscape of the PD-1/PD-L1 mechanistic role in tumor immune evasion and therapeutic outcome for cancer treatment. We also review the current progress in clinical trials, combination of drug therapy with immunotherapy, safety, and future of check point inhibitors for multiple types of cancer…

PD-1 associated immune-resistance depends on the accessibility of PD-L1 ligand in the tumor. The PD-L1 expression is monitored either by upregulation of PI3K-Akt kinases or secretion of IFN-γ, and due to PD-L1 expression, variability in two general types of immune resistance is observed, namely, (I) innate immune resistance, and (II) adaptive immune resistance (not to be confused with innate and adaptive immunity.

With innate immune resistance, in glioblastomas the PD-L1 expression is driven by downregulation of PTEN which is linked to activation of PI3K-Akt tumorigenic signaling. Similarly, the unresponsiveness of PD-1 blockade therapy in prostate cancers has been

attributed to the PD-L1 mediated innate immune resistance. Certain lymphomas and lung cancers have been reported to drive PDL1 expression through the upregulation of the signal transducer and activator of transcription 3 (STAT3) and lymphoma kinase (ALK) signaling resistance. The STAT3 activation is modulated through pro-inflammatory cytokines, such as IL-6 and the IL-6-STAT3 axis is considered as one of the crucial pathway in tumorigenic macrophage polarization and immune suppression.

In adaptive immune resistance, in some tumors, the PD-L1 expression is induced due to the secretion of pro-inflammatory IFN-γ from tumor and tumor-stromal cells that neutralize CD8+ cytotoxic T cell induced anti-tumor immune responses. The adaptive immune response in various preclinical and clinical studies represents an alternative mechanism of conventional drug resistance that involves the mutation of the drug targets.

The authors now discuss several of the above:

The presence of PD-1 and PD-L1 has a major role in the inhibition of effector T-cell function.

Clinical studies have indicated that antibodies blocking PD-1 and PDL1 have a reliable effect on many advanced malignancies. PD-1 and PD-L1 targeting is an efficient way to maintain the function of effector T-cells.

Monoclonal antibodies (mAbs) are a class of drugs called checkpoint inhibitors that inhibit the interaction of PD-1 and PD-L1 and overcome the disadvantages of conventional anticancer therapy. In vitro and in vivo studies that were done by Lussier et al. showed that blocking PD-1 using an antibody can partially enhance T-cell function. MAbs can significantly reduce toxicity well within tolerable limits, while being able to shrink solid tumors, suppress advanced tumors and metastasis, and overall improve patient survival.

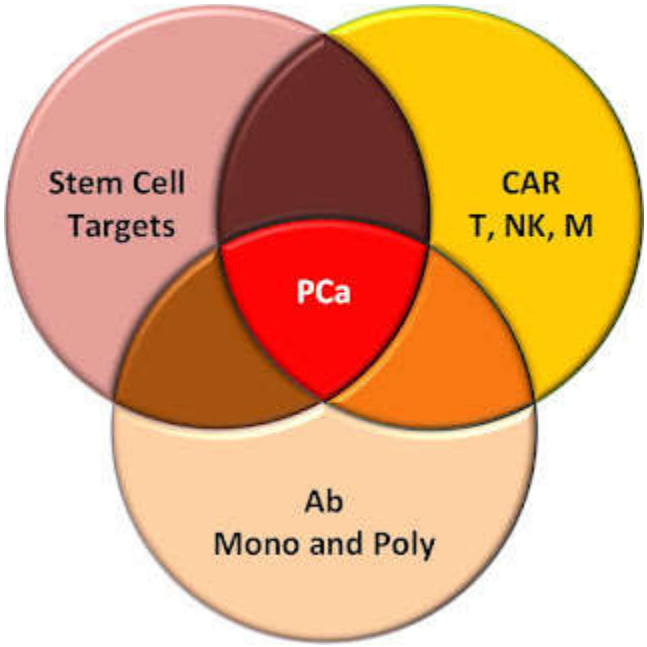
Hundreds of clinical trials on anti-PD-1 and PD-L1 mAbs are under active development. Some of them having entered phase 3 clinical trials and are benefiting many patients. The FDA has recently granted approval to some anti-PD-1and PD-L1 mAbs targeting a range of human cancers. The clinical activity of anti-PD-1and PD-L1 mAbs holds promise in targeting PD-1 and PD-L1 immune checkpoints, thereby ameliorating patient conditions significantly.

The anti-PD-1 therapies approved by the FDA and under active clinical trials for renal cell carcinoma, NSCLC (non-small cell lung carcinoma), HNSCC (head and neck squamous cell carcinoma), and bladder (urothelial) cancer are summarized below and noted in Tables 1, 2. The PD-1 and PD-L1 is a receptor-ligand system and in tumor microenvironment they are attached to each other, resulting blockade of anti-tumor immune responses. PD-1 is majorly expressed on the T cells of the immune system, whereas PD-L1 is on the cancer cells and antigen- presenting cells. Therefore, the inhibitors that block the interaction of PD-1 and PD-L1 will cause resurrection of T-cell mediated anti-tumor immune effect. The PD-1 and PD-L1 antibody inhibitors have been designed to block either the PD-1 or the PD-L1 side and turn on T-cell mediated immunity. Currently, it is not clear whether the PD-1 and PDL1 inhibitors are more effective.

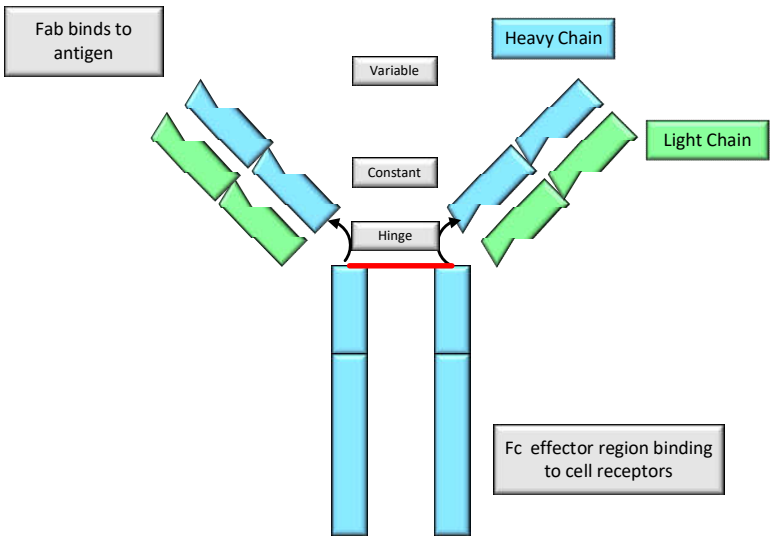
The effectiveness of PD-1 and PD-L1 inhibitors depends on patients' characteristics, such as (i) gender, (ii) types of tumors, (iii) mutation, translocation of genes (EGFR, Kras, ALK), and (iv) metastases of tumor. As tumor is heterogeneous in nature, the expression of PD-L1 is not uniform, thus PD-L1 immunohistochemistry staining varies with tumor locations.

Therefore, indication of PDL1 expression and response of PD-L1 inhibitors remain debatable and needs to be understood deeply. Similarly, PD-1 expression also depends of tumor patient's characteristics Immune response: The phase I studies with anti-PD-1 drugs, such as Nivolumab and Pembrolizumab with non-small-cell lung cancer, advanced melanoma, renal cell carcinoma, and other solid tumor patients have demonstrated very promising response with minimal side effects. Inspired from phase I response, PD-1 blockers were studied for further trials and in phase III trial patients with advance melanoma showed excellent response than the NSCLC, RCC.

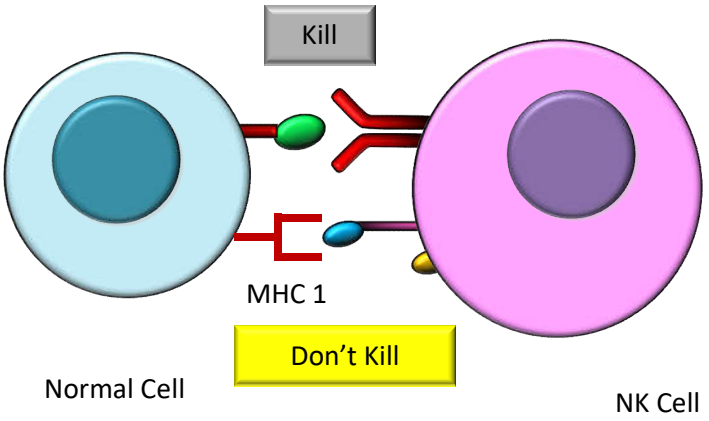
Consider a cancer cell with the surface markers as shown below. This is quite common for PCa cells, especially stem cells. We thus can look at the approaches below. First identify and find markers on the stem cells. The choose a CAR approach with one of several immune cells and/or choose an antibody approach using what are called poly specific antibodies.

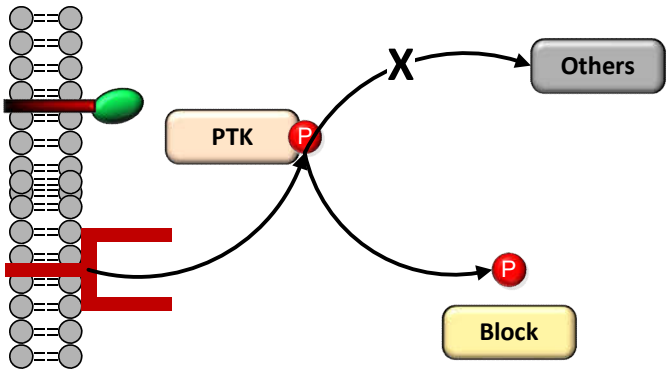


Now antibodies, Ab, are common elements of the immune system.

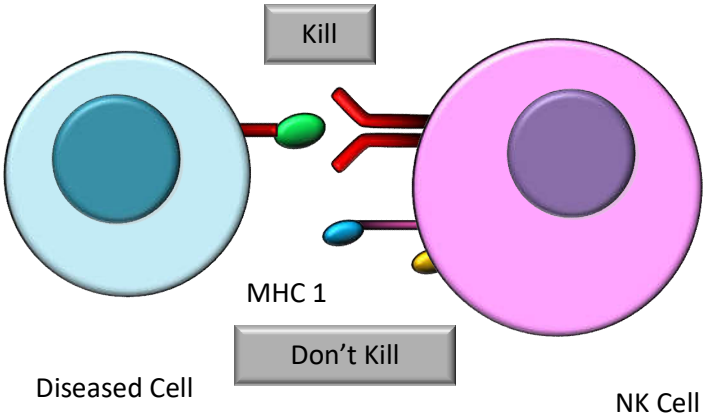


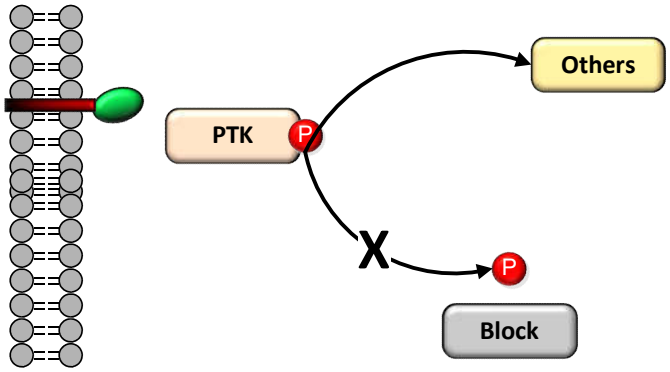
As shown above they have a collection of proteins linked so that at the short end they attach to the target cell surface marker and at the lower end to an immune system cell. The result is elimination of the target. Think COVID. An example is below with an NK or natural killer cell. If a normal cell is found then it will have a surface marker MHC saying it is a good cell so do not do anything.





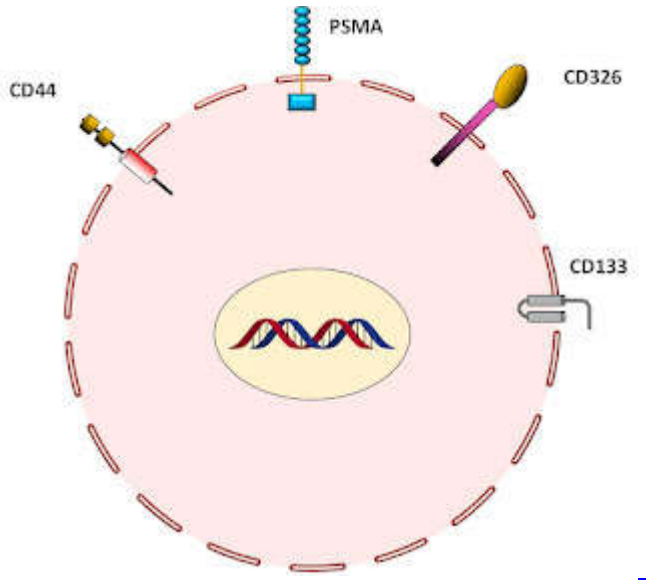
However, if MHC is absent we get what we see below:



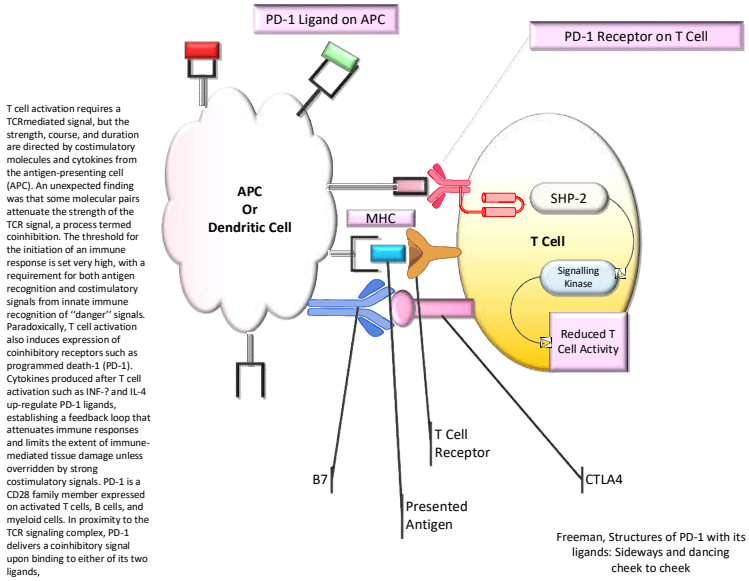


which results in an attack and elimination of the bad cell.

Thus, we rely upon these Ab to help direct the immune cells to kill the bad or cancer cells. There are two possible ways to do this. One is to create Ab to attach to the cell and then wait for immune cells to attack. Or we can create immune cells with the ligand which can attach to the cancer cell. These are CAR or chimeric cells. The malignant prostate cell typically has these 4 if not more targets as shown below.



The graphic below depicts the interaction between an APC and a T cell. Recall that MHC I are on all somatic cells. Furthermore, the selection of "self" MHCs is performed in the thymus (see Abbas et al) This the presentation of an AG on an APC begins the process. Note PD-1 can block the T cell but if we can block PD-1 then the T cell can effect itself. This was one of the first approaches to immunotherapy.



T cell activation requires a TCRmediated 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,

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

3 IMMUNE SYSTEM TARGETS

To effect efficacy in a vaccine against cancers or frankly any pathogenic entity one needs an antigen, a target, for the immune system to identify the pathogenic entity. As Liu et al (2022) have noted:

Antigen selection is a critical process of cancer vaccines design. Tumor antigens recognized by T lymphocytes are central to the efficacy of cancer vaccines. The ideal antigen for a cancer vaccine should be highly immunogenic, explicitly expressed in all cancer cells (not in normal cells) and necessary for the survival of cancer cells.

Tumor antigens can be divided into TAAs (tumor associated antigens) and TSAs (tumor specific antigens).

TAAs also be known as tumor-shared antigens. TAAs include "self-antigens" such as differentiated antigens, overexpressed antigens, cancer-testicular antigens, and viral-original "non-self" antigens.

Prominent examples of overexpressed tumor antigens are human epidermal growth factor receptor 2 (HER2) and human telomerase reverse transcriptase . Tissue differentiation antigens are expressed by tumor cells and normal cells of the same tissue origin as tumor cells, such as prostate-specific antigen (PSA) expressed in the prostate gland and prostate cancer, melanoma antigens tyrosinase expressed by normal melanocytes, and melanoma cells. TAAs are adaptable and can be applied to different patients. Early cancer vaccines were primarily focused on TAAs. However, due to the central immune tolerance of the thymus, activated T cells that recognize TAAs or other autoantigens may be eliminated during development, which will affect the efficacy of the vaccine.

Thus, cancer vaccines that use TAAs must be compelling enough to "break the tolerance." Although TAAs have been focused on for many years, clinical trials of cancer vaccines based on TAAs have had limited success. In addition, TAAs are also expressed in nonmalignant tissues, increasing the risk of vaccine-induced autoimmune toxicity.

TSAs are a class of proteins specifically expressed in tumor cells.

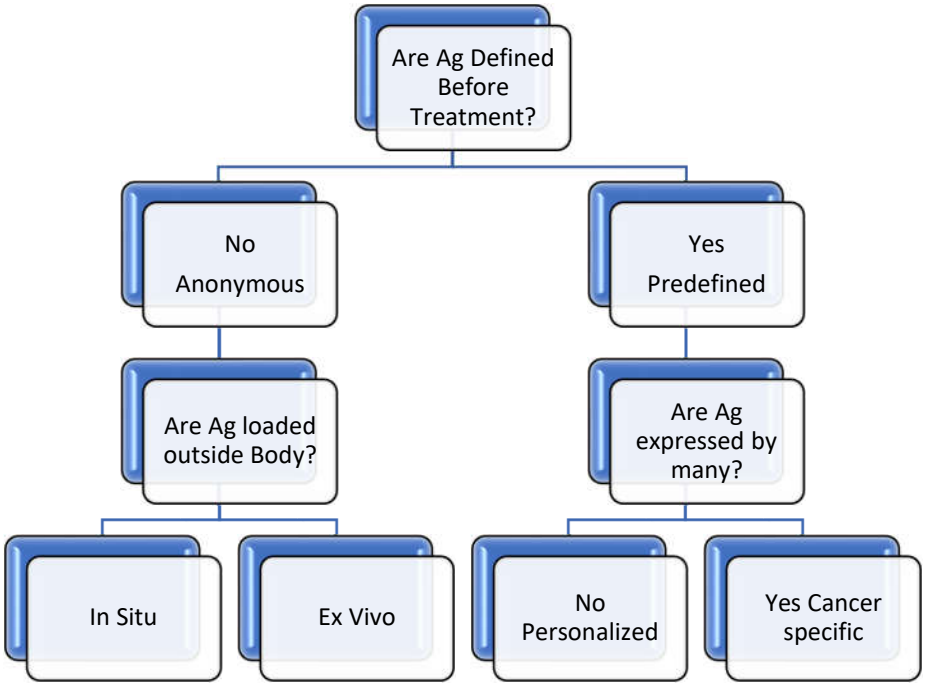
TSAs are mentioned as neoantigens sometimes. The individual-specific non-autogenous proteins produced due to mutations in tumor cells ***are called neoantigens****. Neoantigens are expressed only by tumor cells, triggering a valid tumor-specific T-cell response with limited "off-target" damage. Compared with TAAs, neoantigens have more potent immunogenicity and higher major histocompatibility complex (MHC) affinity. What's more, they are unaffected by central immune tolerance. The wide application of nextgeneration sequencing technology makes it possible to identify personalized neoantigens in a timely and cost-effective manner. Further, the development of algorithms for predicting MHC I class binding epitopes has also greatly facilitated the discovery of potential new immunogenicity epitopes.*

Cancer vaccines targeting neoantigen have become the main direction of tumor vaccine in recent years. Recently, several clinical trials evaluating neoantigen vaccines have yielded promising results with improved patient survival. An mRNA neoantigen melanoma vaccine is a typical example that induced T cell infiltration and neoantigen-specific killing of autologous tumor cells. The incidence of metastatic events was significantly reduced after vaccination, resulting in sustained progression-free survival

Lin et al (2022) have noted:

We consider ***two types of tumor-specific antigens (TSAs), including viral antigens and neo-epitopes resulting from non-synonymous somatic mutations****, and* ***two types of tumor-associated antigens (TAAs), including tissue-specific antigens and development-specific antigens****.*

We show below the classification discussed above.



All the vaccines discussed might mobilize T cell responses against both TSAs and TAAs, except for predefined personalized antigen vaccines, which generally use TSAs. In this latter case, it is possible that hotspot mutations in cancer-related genes could be present in the tumors of different patients sharing human leukocyte antigen (HLA) molecules. The uptake of tumor antigens by APCs is a critical event.

A majority of TAAs are intracellular and thereby difficult to target with humoral responses or derived therapies such as monoclonal antibodies, CAR T cells or bispecific T cell engagers.

Although intracellular TAAs can be detected by TAA-specific T cells through HLA molecules on tumor cells, deficits in tumoral costimulatory molecules generally yield T cell anergy or exhaustion. Therefore, APCs, particularly DCs, are essential for anti-tumor T cell priming.

The cDC1 (type 1 conventional DC) subset (or Batf3-dependent CD103 +XCR1 +CD141 +Clec9A + DCs) is specifically capable of cross-presentation: taking up exogenous antigens and presenting them on HLA-I to CD8+ T cells. Therefore, by activating tumor antigen-loaded DCs, cancer vaccines may induce immune responses against a large array of intracellular antigens. From this perspective, the different vaccine types differ merely by methods of colocalizing tumor antigens with cross-presenting DCs.

Predefined antigens can be further classified by the frequency of expression across patient cohorts.

Shared antigens are those expressed in a sufficient proportion of patients such that vaccinologists can target these patient groups (frequently within patient subsets of tumor types) using standard testing.

Shared antigen vaccines can thus target both TSAs and TAAs. *As examples, the neo-epitope TSA epidermal growth factor receptor variant III (EGFRvIII) is expressed in ~25% of EGFR-overexpressing glioblastomas (GBMs) and the viral TSA human papilloma virus E6 and E7 proteins (HPV E6 and E7) are expressed in ~60% of oropharyngeal cancers and nearly all cervical cancers, whereas the TAA Wilms' tumor protein (WT1) is overexpressed in most acute myeloid leukemias (AMLs), breast cancers and Wilms' tumors.*

Shared antigen vaccines are distinguished from personalized antigen vaccines in that the former can be assessed with standard testing such as cytology, immunohistochemistry and flow cytometry. Predefined, shared antigen vaccines have been the primary focus of preclinical and clinical research since the 1990s and have provided foundational lessons. Personalized antigens are unique to the vaccinated patient.

Personalized antigen vaccines have developed alongside the modern era of high-throughput gene sequencing and generally consist of TSA neo-epitopes that, in contrast to the shared TSA EGFRvIII or Kirsten rat sarcoma virus (KRAS)G12D, are not sufficiently common to target a large group of patients.

This approach allows the immune system to target tumors lacking known shared antigens but also places a burden on the vaccinologist to iteratively determine the optimally immunogenic epitopes. Immunogenic epitopes must bind with sufficient avidity to both the peptide groove of an HLA molecule and to the complementarity-determining regions of a reactive T cell receptor (TCR). Peptide–HLA (and, to a lesser degree, TCR) avidities can be modeled and estimated in silico for an individual patient's tumor mutanome, although these algorithms are still improving.

Such approaches also pose a logistical burden of biopsying tumors for exome and RNA sequencing or for proteomic analysis of peptides actually presented by patient HLA class I

molecules. These techniques also require time and resources inherent in vaccine design and subsequent personalized neo-epitope pool production.

3.1 TAA

Buonaguro and Tagliamonte note:

Cancer cells, as result of their malignant profile, can constitutively overexpress antigens derived from protein, which are mainly involved in the replication and/or migration of the cancer cells.

The antigens derived from the aberrantly overexpressed self-antigens in tumor cells compared to normal cells (e.g., ***RAGE-1, hTERT, HER2, mesothelin, and MUC-1****) are* ***defined as tumor-associated antigens (TAAs) and might represent universal antigens among patients with the same malignancy****.*

Besides the overexpressed antigens, TAAs can include:

cell lineage differentiation antigens*, which are normally not expressed in adult tissue (e.g.,* ***tyrosinase, gp100, MART-1, prostate-specific antigen (PSA)****;*

*prostatic acid phosphatase (****PAP****));*

and ***cancer/germline antigens*** *(also known as cancer/testis), which are normally expressed only in immune privileged germline cells (e.g.,* ***MAGE-A1, MAGE-A3, NY-ESO-1, and PRAME****).*

Overexpressed and tissue differentiation antigens are able to induce an antitumor immune response when high levels of expression of these proteins reach the threshold for T cell recognition, breaking immunological tolerance.

However, the main drawback with using TAAs in cancer immunotherapy is the potential induction of autoimmunity against the corresponding normal tissues.

As these antigens are also expressed in healthy tissue as self-antigens, they are generally characterized by low immunogenicity, and T cells have low affinity receptors (TCR), which are unable to mediate effective anti-tumor responses. Additionally, T cells that recognize these antigens may be removed from the immune repertoire by central and peripheral tolerance.

The formulation with an effective adjuvant may overcome the problem, significantly increasing the immunogenicity of the antigens and resulting in a clinical benefit for cancer patients.

3.2 TSA

TSA are built upon tumor specific antigens. Namely based upon patient tumor cells sequenced and specific Ag observed. As Liu et al (2022) note:

The high-quality neoantigens should be associated with the following features:

First, they should manifest strong binding affinity to human leukocyte antigen (HLA);

second, they should be highly heterologous compared to the wild type;

third, they can be expressed by most tumor cells;

fourth, they are generated as the consequences of mutations that affect survival.

The neoantigens with these features could induce a robust immune response and prevent the development of tumor-immune escape.

Currently, no studies have shown the optimal number of neoantigens for a tumor vaccine. A neoantigen vaccine usually contains several to dozens of neoantigens. For example, a personalized neoantigen DNA vaccine (GNOS-PV02) encodes up to ***40 neoantigens****, including all detected neoantigens for the majority of hepatocellular carcinoma patients. In recent years, to increase the vaccine's effectiveness, scientists have combined shared antigens with neoantigens to expand the antigen pool for vaccination.*

For example, the APVAC1/2 vaccines, which contain shared tumor antigens and patient specific neoantigen, can effectively activate the T-cell response in the treatment of glioblastoma. Furthermore, early clinical studies of personalized neoantigen vaccines combined with PD-1 or PD-L1 inhibitors have also shown anti-tumor activity.

3.3 TARGET PROCESS

As Liu et al (2022) note:

Mature DCs present the processed antigen epitopes on MHC I and MHC II molecules to naive CD4+ and CD8+ T cells.

Moreover, DCs also secrete IL-12 and interferon-γ (IFN-γ) to increase costimulatory factor production. Tumor-specific T cells are activated by binding to MHC–peptide complex–T cell receptor and costimulatory "signal 2".

Activated T cells then differentiate into long-lived memory T cells and effector T cells. Effector tumor-specific T cells amplify and are trafficked to TME to induce tumor killing through cytotoxicity and the production of effector cytokines. In addition, activated B cells promote tumor apoptosis through antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity.

Further, immunogenic cell death release tumor antigens and damage-associated molecular patterns. In turn, the tumor antigens released by lysed tumor cells can be captured, processed, and presented again by APCs to induce polyclonal T cell responses, thereby increasing the antigenic breadth of anti-tumor-immune responses. These processes are known as the cancer-immunity cycle

Saxena et al note the factors to be sought in neo-antigens:

Foreignness *The greater the similarity to the wild-type amino acid sequence, the higher the probability of the responding T cells to be deleted during thymic selection*

Clonal distribution *Subclonal mutations are present in a small percentage of tumour cells and have high chance of losing expression either spontaneously or after ICI*

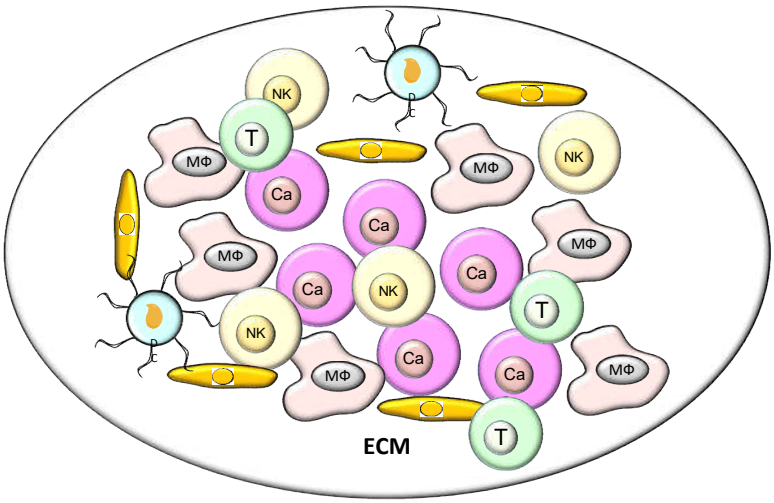
Driver vs passenger mutation *Passenger mutations are subject to loss of expression through tumour evolution or immune resistance. Driver mutations are more conserved as these serve critical survival functions*

MHC presentation *Neoantigen presentation on MHC class I and/or MHC class II molecules and expression in tumours with higher HLA heterozygosity in HLA class I loci is more likely to induce T cell infiltration and increase survival in response to ICI*

TCR avidity *• High TCR avidity of neoantigens induces a strong CTL-driven response to treatment • Hard to predict*

4 TUMOR MICROENVIRONMENT

The tumor micro environment, TME, is the complex of cells that agglomerate with the tumor cells and support and protect them. An example graphic is shown below. The amalgam of cells is further set in a protective environment termed the extracellular matrix. We will examine these next.



As Anderson and Simon recently noted:

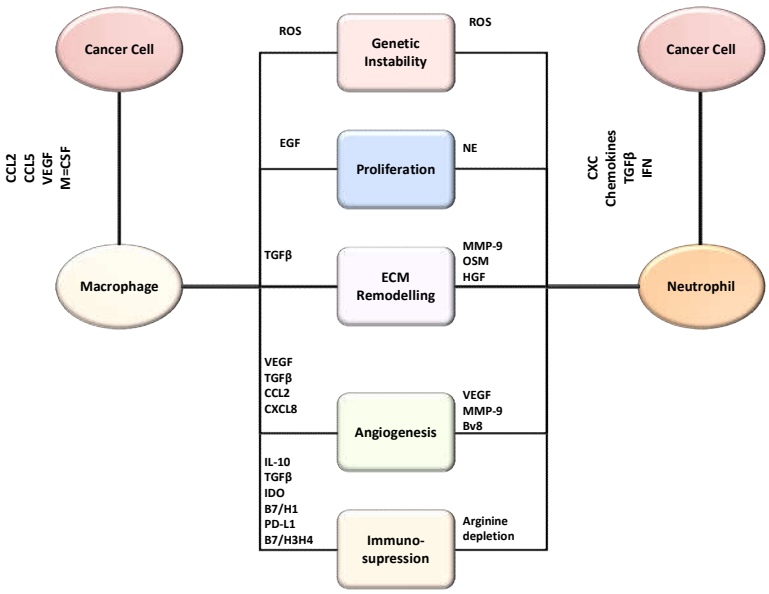
A tumor is not simply a group of cancer cells, but rather a heterogeneous collection of infiltrating and resident host cells, secreted factors and extracellular matrix. Tumor cells stimulate significant molecular, cellular and physical changes within their host tissues to support tumor growth and progression. An emerging tumor microenvironment is a complex and continuously evolving entity.

The composition of the tumor microenvironment varies between tumor types, but hallmark features include immune cells, stromal cells, blood vessels, and extracellular matrix. It is believed that the "tumor microenvironment is not just a silent bystander, but rather an active promoter of cancer progression*".*

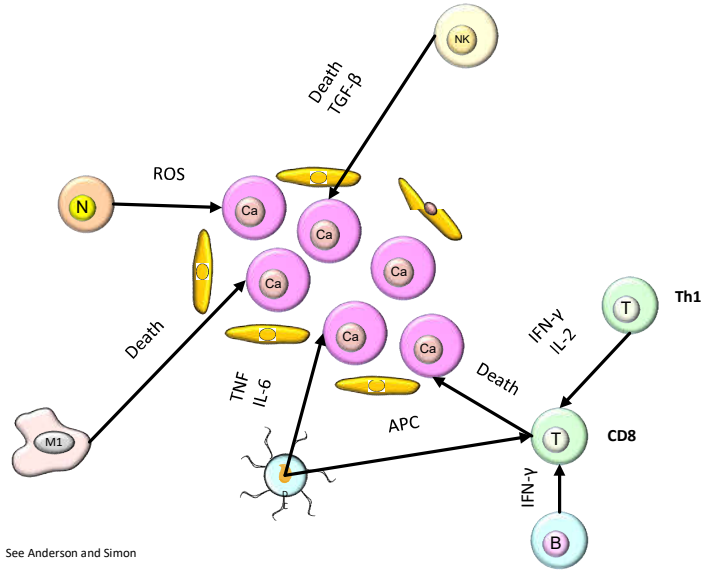
Early in tumor growth, a dynamic and reciprocal relationship develops between cancer cells and components of the tumor microenvironment that supports cancer cell survival, local invasion and metastatic dissemination. To overcome a hypoxic and acidic microenvironment, the tumor microenvironment coordinates a program that promotes angiogenesis to restore oxygen and nutrient supply and remove metabolic waste.

Tumors become infiltrated with diverse adaptive and innate immune cells that can perform both pro- and antitumorigenic functions. An expanding literature on the tumor microenvironment has identified new targets within it for therapeutic intervention.

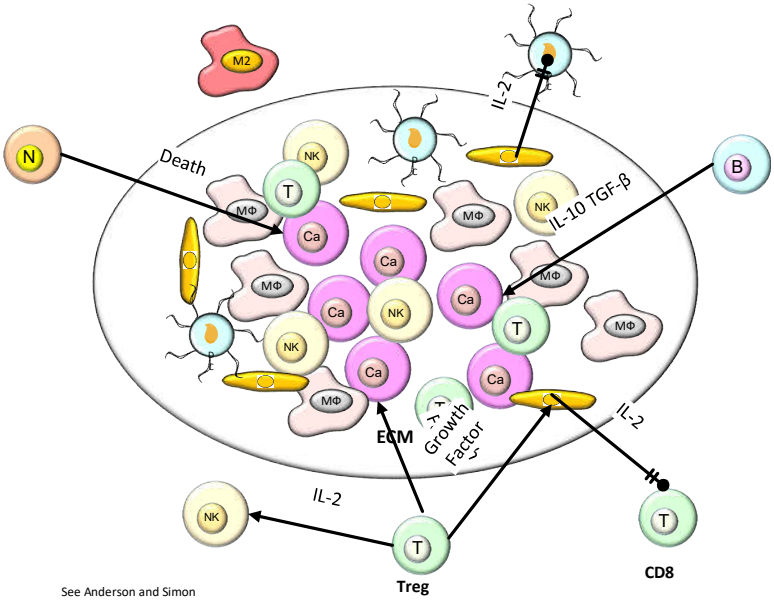
The figure below demonstrates some of the complexity of that signalling.



On the tumor suppressor side one can see the following interactions"



Yet on the tumor supporting side we have the following:

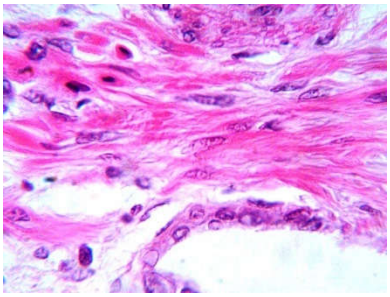


We will now discuss some of these factors. Cancer vaccines must deal with this complex environment. It is not just attacking the cancer cell but getting to it in the first place. The TME can be a highly protective and supportive medium.

4.1 Extracellular Matrix (ECM)

The extra cellular matrix, ECM, is a complex of proteins which occupy the space outside of the cell and provide a "structure" to the cellular complex. In contrast to the TME, the ECM is an amalgam of supporting elements that create in conjunction with cells a stable homeostatic element in the human body. However, the ECM like the TME can be hijacked by the cancer cells and Thus, it is essential to understand its functioning.

We start with some recent work on the ECM. The image below is from a prostate slide and the gland is at the bottom and the ECM fibers are above.



From Kumar et al we have:

The ECM is a network of interstitial proteins that constitutes a significant proportion of any tissue.

Cell interactions with ECM are critical for development and healing, as well as for maintaining normal tissue architecture. Much more than a simple "space filler" around cells, ECM serves several key functions:

1. *Mechanical support for cell anchorage and cell migration, and maintenance of cell polarity.*
2. *Control of cell proliferation, by binding and displaying growth factors and by signaling through cellular receptors of the integrin family. The ECM provides a depot for a variety of latent growth factors that can be activated within a focus of injury or inflammation.*
3. *Scaffolding for tissue renewal. Because maintenance of normal tissue structure requires a basement membrane or stromal scaffold, the integrity of the basement membrane or the stroma of parenchymal cells is critical for the organized regeneration of tissues. Thus, ECM disruption results in defective tissue regeneration and repair, for example, cirrhosis of the liver resulting from the collapse of the hepatic stroma in various forms of hepatitis.*
4. *Establishment of tissue microenvironments. The basement membrane acts as a boundary between the epithelium and underlying connective tissue; it does not just provide support to the epithelium but is also functional, for example, in the kidney, forming part of the filtration apparatus.*

Cell surface integrins interact with the cytoskeleton at focal adhesion complexes (protein aggregates that include vinculin, α-actinin, and talin. This can initiate the production of intracellular messengers or can directly transduce signals to the nucleus. Cell surface receptors for growth factors can activate signal transduction pathways that overlap with those mediated through integrins. Signals from ECM components and growth factors can be integrated by the cells to produce a given response, including changes in proliferation, locomotion, and/or differentiation.

The ECM is constantly being remodeled; its synthesis and degradation accompany morphogenesis, tissue regeneration and repair, chronic fibrosis, and tumor invasion and metastasis. ECM occurs in two basic forms: interstitial matrix and basement membrane

1. Interstitial matrix is present in the spaces between cells in connective tissue, and between the parenchymal epithelium and the underlying supportive vascular and smooth muscle structures. The interstitial matrix is synthesized by mesenchymal cells (e.g., fibroblasts), forming an amorphous three-dimensional gel. Its major constituents are fibrillar and nonfibrillar collagens, as well as fibronectin, elastin, proteoglycans, hyaluronate, and other constituents.

2. Basement membrane. The seemingly random array of interstitial matrix in connective tissues becomes highly organized around epithelial cells, endothelial cells, and smooth muscle cells, forming the specialized basement membrane. This is synthesized conjointly by the overlying epithelium and the underlying mesenchymal cells, forming a flat lamellar "chicken wire" mesh (although labeled as a membrane, it is quite porous). The major constituents are amorphous nonfibrillar type IV collagen and laminin.

The components of the ECM fall into three groups of proteins:

1. *Fibrous structural proteins such as collagens and elastins that confer tensile strength and recoil*

2. *Water-hydrated gels such as proteoglycans and hyaluronan that permit compressive resistance and lubrication*

3. *Adhesive glycoproteins that connect ECM elements to one another and to cells*

As Liu et al note, the ECM has that "soil" like quality:

Tumor cells reside in a highly complex and heterogeneous tumor microenvironment (TME), which is composed of a myriad of genetically stable non-cancer cells, including fibroblasts, immune cells, endothelial cells, and epithelial cells, and a tumor-specific extracellular matrix (ECM).

Cancer-associated fibroblasts (CAFs), as an abundant and active stromal cell population in the TME, function as the signaling center and remodeling machine to aid the creation of a desmoplastic tumor niche. Although there is no denial that the TME and CAFs may have anti-tumor effects as well, a great deal of findings reported in recent years have convincingly revealed the tumor-promoting effects of CAFs and CAF-derived ECM proteins, enzymes, chemical factors and other downstream effectors.

While there is growing enthusiasm for the development of CAF-targeting therapies, a better understanding of the complexities of CAF-ECM and CAF-cancer cell interactions is necessary before novel therapeutic strategies ***targeting the malignant tumor "soil" can be successfully implemented in the clinic.***

The focus on intracellular pathways has been a prime direction of research in the development of cancers. However, there has from time to time been some focus on the extracellular matrix, the "ECM", which relates in many ways to the stability of the cell, its localization. Cancer cells lose this sense of localization and begin to move.

The processes at play in the ECM have a significant impact on the processes that occur within a cell. Thus, it is essential to have an understanding of the ECM. Recent work by Fisher and his people on MDA-9, a controller of certain ECM elements, demonstrates a control path that influences the internal pathways. We discuss the ECM in the context of the MDA-9 developments.

In this section we use a recent development in understanding the impact of Mda-9 and the nexus with the extra cellular matrix, ECM, and the control of metastatic melanoma.

We first review the Fisher Team efforts as recently presented and then we examine the standard intracellular pathways that have been examined and from that we provide an overview of the

extra cellular matrix, ECM, which is the "glue" binding together cells and facilitating cell to cell communications.

We find this an interesting focus or research for several reasons:

1. It examines the ECM which has received limited focus.

2. It focuses on pathways as we have been also doing and specifically an interesting adjunct to the current B-RAF approach.

3. It establishes a clear path forward which is logically and experimentally based and verifiable.

There has been limited prior research on these issues. In Hearing and Leong, 380-386, there is a limited discussion regarding the ECM and melanoma with references. The work by Zent and Pozzi provides a broad and detailed perspective of the ECM with many cancers. However, their work is not specific to melanoma. In Weinberg there are references but there does not appear to be any singular focus on the ECM as a standalone system element.

As NCBI states[7]:

Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases.

The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling

The ECM has often been neglected when discussing cancer pathways. Weinberg has multiple references but does not seem to place it in any specific spotlight. In Lewin, Cell[8], the discussion is quite well focused but yet there is but passing reference to the impact on cancer pathways. Specifically, there is reference to MMP-9[9], here a metalloproteinase, and melanoma[10].

---

[7] http://www.ncbi.nlm.nih.gov/gene/4318

[8] See Cassimeris, L., et al, Lewin's Cell, 2nd Ed, Jones and Bartlett (Boston) 2011.

[10] As NCBI states: "*Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling.*" see http://www.ncbi.nlm.nih.gov/gene/4318

The ECM is the collection of molecules that lie between the cell walls. The ECM provides for structural integrity as well as facilitates and even participates in cell-to-cell communications. The ECM is a highly complex and quite active element in the ongoing life of the cells. In addition, we all too often look to what happens in a cell, with at best a nod to ligands, and we do not look at the cell internals as well as the ECM as a holistic system totality. The work of the Fisher Team in a small way may help refocus this effort on the complex as a working whole.

4.1.1 Collagen

Collagens provide structure support. They are triple helical proteins wrapped to provide that supporting structure between the cells. There any many types of collagen and the actually assembly commences within the cell and the semi-finished product passes through the cell wall to the ECM. For our purposes the collagen complexes are at this time of limited interest. From Kumar et al we also have:

Collagens are composed of three separate polypeptide chains braided into a ropelike triple helix. About 30 collagen types have been identified, some of which are unique to specific cells and tissues. Some collagen types (e.g., types I, II, III, and V collagens) form linear fibrils stabilized by interchain hydrogen bonding; such fibrillar collagens form a major proportion of the connective tissue in structures such as bone, tendon, cartilage, blood vessels, and skin, as well as in healing wounds and scars.

The tensile strength of the fibrillar collagens derives from lateral crosslinking of the triple helices by covalent bonds, an unusual post-translational modification that requires hydroxylation of lysine residues in collagen by the enzyme lysyl oxidase. Because lysyl oxidase is a vitamin C-dependent enzyme, children with ascorbate deficiency have skeletal deformities, and people of any age with vitamin C deficiency heal poorly and bleed easily because of "weak" collagen. Genetic defects in collagens cause diseases such as osteogenesis imperfecta and certain forms of Ehlers-Danlos syndrome.

Nonfibrillar collagens variously contribute to the structures of planar basement membranes (type IV collagen); help regulate collagen fibril diameters or collagen-collagen interactions via so-called "fibril-associated collagen with interrupted triple helices" (FACITs, such as type IX collagen in cartilage); and provide anchoring fibrils within basement membrane beneath stratified squamous epithelium (type VII collagen).

4.1.2 Elastin.

From Kumar et al we have the following discussion on elastin:

The ability of tissues to recoil and recover their shape after physical deformation is conferred by elastin. Elasticity is especially important in cardiac valves and large blood vessels, which must accommodate recurrent pulsatile flow, as well as in the uterus, skin, and ligaments. Morphologically, elastic fibers consist of a central core of elastin with an associated meshlike network composed of fibrillin. The latter relationship partially explains why fibrillin defects lead

to skeletal abnormalities and weakened aortic walls, as in individuals with Marfan syndrome. Fibrillin also controls the availability of TGF-β.

4.1.3 Fibronectin

Fibronectin facilitates the process of connecting cells to matrices of collagen. Fibronectin proteins have a six-element structure. Cells bind to fibronectin via receptors called integrins. The fibronectin binding Thus, activates pathways within the cell, thereby establishing an intra and intercellular pathway complex. The pathways activated control growth, movement and cell differentiation.

We can now examine some of the relevant literature on fibronectin and melanomas. As Yi and Ruoslahti state:

Fibronectin is a prototypic extracellular matrix (ECM) protein that is deposited by various types of cells into an adhesive fibrillar meshwork of protein.

Fibronectin, and ECM in general, control many cellular functions, including growth, migration, differentiation, and survival.

The signals that control these behaviors are transmitted from the ECM to the cell by integrins, a family of transmembrane receptors. Malignant cells often bypass the ECM–integrin signaling system; they are not bound by the spatial constraints imposed by the ECM on normal cells, and they no longer require ECM contact for survival

Namely fibronectin is a broad-based controller of many cellular processes. Understanding them may open options for therapeutics. Liu et al state:

Tumor cells frequently exhibit decreased adhesiveness due to failure to deposit stromal fibronectin (FN), permitting more rapid proliferation, migration, invasion, and metastasis. Although up-regulation of FN has been noted in gene profiles of carcinomas compared with normal tissue, reduced FN expression has been described at the peripheral margins of invading tumors. In this study, we investigate the role of FN in cancer behavior. …

Loss of spatial stability is a common feature of many malignancies. Cells proliferate and the loss of structure characteristic result in disoriented masses of the new cells as they multiply.

Neoplastic transformation is often characterized by changes in the organization of the cytoskeleton, decreased cell adhesion, and aberrant adhesion–mediated signaling. Disruption of normal cell adhesion contributes to enhanced proliferation, migration, and invasion leading to metastasis. Fibronectin (FN) is an extracellular matrix protein with putative roles in mediating these actions. Indeed, tumor cells with decreased adhesiveness frequently fail to deposit stromal FN.

In particular, reduced FN expression has been noted in transformed cell lines and primary tumors, including thyroid cancer, where diminished FN has been identified at the periphery of

invasive tumor margins. In this context, we found that down-regulation of FN stimulates thyroid cancer cell proliferation and tumor growth.

Conversely, 1, 25-dihydroxy vitamin D3 treatment increases cell adhesiveness and inhibits cell proliferation and tumor growth through enhanced FN expression.

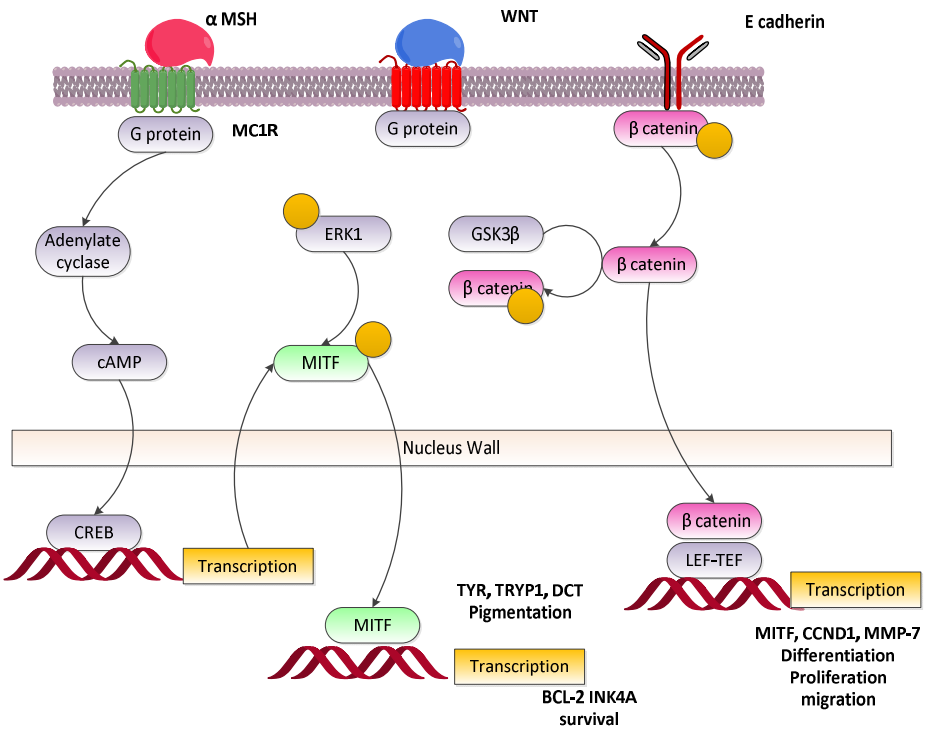
From Kumar et al we also have:

This is a large (450 kD) disulfide-linked heterodimer that exists in tissue and plasma forms; it is synthesized by a variety of cells, including fibroblasts, monocytes, and endothelium. Fibronectin has specific domains that bind to distinct ECM components (e.g., collagen, fibrin, heparin, and proteoglycans), as well as integrins . In healing wounds, tissue and plasma fibronectin provide a scaffold for subsequent ECM deposition, angiogenesis, and reepithelialization.

We will come back to fibronectin in out later analysis.

4.1.4 E-cadherin

We have discussed E-cadherin at length in previous work. It plays a critical role in stabilizing cell adhesion and localization. Loss of E-cadherin results in loss of cell localization and Thus, cell movement. Specifically in melanocytes the cells begin to leave the basal layer and migrate upward as in melanoma in situ and downward as in superficial spreading melanoma.



As Swiatoniowski et al state:

Integrins are molecules which play a significant role in cell-extracellular matrix (ECM) interactions. They interact with the RGD tripeptide of fibronectin (FN), one of the main components of ECM. Labile expression of FN has been proven to play an important role both in the normal developmental process (morphogenetic movements) and in the course of carcinogenesis …

Many authors have implicated loss or decrease of EC expression as an independent negative prognostic marker in breast cancer patients. There is increasing experimental evidence for a relationship between the EC level and different features of breast cancer, including histological grade and axillary lymph node involvement…. In conclusion, our experiment revealed no prognostic value for EC or FN expressions in a homogenous group of patients

4.1.5 Proteoglycan

Proteoglycans are single polypeptide with multiple sugars attached. They provide for hydration in the ECM. From Kumar et al we have the following details:

Proteoglycans form highly hydrated gels that confer resistance to compressive forces; in joint cartilage, proteoglycans also provide a layer of lubrication between adjacent bony surfaces. Proteoglycans consist of long polysaccharides called glycosaminoglycans (examples are keratan sulfate and chondroitin sulfate) attached to a core protein; these are then linked to a long hyaluronic acid polymer called hyaluronan in a manner reminiscent of the bristles on a test-tube brush. The highly negatively charged, densely packed sulfated sugars attract cations (mostly sodium) and abundant water molecules, producing a viscous, gelatin-like matrix. Besides providing compressibility to tissues, proteoglycans also serve as reservoirs for secreted growth factors (e.g., FGF and HGF). Some proteoglycans are integral cell membrane proteins that have roles in cell proliferation, migration, and adhesion, for example, by binding and concentrating growth factors and chemokines.

4.1.6 Protease

The proteases are ECM proteins which function to degrade the refuse in the ECM. The metalloproteinases are a family of proteases. They are also called MMP. MMP-9 and MMP-2 are ones of the MMPs often associated with melanoma.

There has been extensive work examining the MMPs and melanoma some dating back to the 1990s, see that of Luca et al. A recent result by Hoffman et al state:

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are involved in tumour progression and metastasis. In this study, we investigated the in vitro and in vivo expression patterns of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 mRNA and protein in a previously described human melanoma xenograft model.

This model consists of eight human melanoma cell lines with different metastatic behaviour after subcutaneous (s.c.) injection into nude mice. MMP-1 mRNA was detectable in all cell lines by reverse transcription polymerase chain reaction (RT-PCR), but the expression was too low to be

detected by Northern blot analysis. No MMP-1 protein could be found using Western blotting. MMP-2 mRNA and protein were present in all cell lines, with the highest expression of both latent and active MMP-2 in the highest metastatic cell lines MV3 and BLM. MMP-3 mRNA was expressed in MV3 and BLM, and in the non-metastatic cell line 530, whereas MMP-3 protein was detectable only in MV3 and BLM.

None of the melanoma cell lines expressed MMP-9. TIMP-1 and TIMP-2 mRNA and protein, finally, were present in all cell lines. A correlation between TIMP expression level and metastatic capacity of cell lines, However, was lacking. MMP and TIMP mRNA and protein expression levels were also studied in s.c. xenograft lesions derived from a selection of these cell lines.

RT-PCR analysis revealed that MMP-1 mRNA was present in MV3 and BLM xenografts, and to a lesser extent in 530. Positive staining for MMP-1 protein was found in xenograft lesions derived from both low and high metastatic cell lines, indicating an in vivo up-regulation of MMP-1. MMP-2 mRNA was detectable only in xenografts derived from the highly metastatic cell lines 1F6m, MV3 and BLM. In agreement with the in vitro results, the highest levels of both latent and activated MMP-2 protein were observed in MV3 and BLM xenografts.

With the exception of MMP-9 mRNA expression in 530 xenografts, MMP-3, MMP-9, and TIMP-1 mRNA and protein were not detectable in any xenograft, indicating a down-regulated expression of MMP-3 and TIMP-1 in vivo. TIMP-2 mRNA and protein were present in all xenografts; interestingly, the strongest immunoreactivity of tumour cells was found at the border of necrotic areas. Our study demonstrates that of all tested components of the matrix metalloproteinase system, only expression of activated MMP-2 correlates with increased malignancy in our melanoma xenograft model, corroborating an important role of MMP-2 in human melanoma invasion and metastasis.

We shall see the impact of MMPs as we examine the pathways.

4.1.7 Integrins

Integrins are for the most part the receptors for ECM proteins. They are one of many such cell surface receptors. The integrins play important roles in cell homeostasis and cell to cell communications. From Kumar et al we also have:

These are a large family of transmembrane heterodimeric glycoproteins composed of α- and β-subunits that allow cells to attach to ECM constituents such as laminin and fibronectin. Thus, functionally and structurally linking the intracellular cytoskeleton with the outside world. Integrins also mediate cell-cell adhesive interactions. For instance, integrins on the surface of leukocytes are essential in mediating firm adhesion to and transmigration across the endothelium at sites of inflammation, and they play a critical role in platelet aggregation. Integrins attach to ECM components via a tripeptide arginine-glycine-aspartic acid motif (abbreviated RGD). In addition to providing focal attachment to underlying substrates, binding through the integrin receptors can also trigger signaling cascades that influence cell locomotion, proliferation, shape, and differentiation.

4.1.8 Laminin

From Kumar et al we have:

This is the most abundant glycoprotein in the basement membrane. It is an 820-kD cross-shaped heterotrimer that connects cells to underlying ECM components such as type IV collagen and heparan sulfate. Besides mediating the attachment to the basement membrane, laminin can also modulate cell proliferation, differentiation, and motility.

4.1.9 MDA-9

Let us briefly examine the gene MDA-9 and its protein Mda-9 and what is known and how it has evolved. Now MDA-9 is located on (8q12). As the NIH data base states:

The protein encoded by this gene was initially identified as a molecule linking syndecan-mediated signaling to the cytoskeleton. The syntenin protein contains tandemly repeated PDZ domains that bind the cytoplasmic, C-terminal domains of a variety of transmembrane proteins. This protein may also affect cytoskeletal-membrane organization, cell adhesion, protein trafficking, and the activation of transcription factors.

The protein is primarily localized to membrane-associated adherens junctions and focal adhesions but is also found at the endoplasmic reticulum and nucleus. Alternative splicing results in multiple transcript variants encoding different isoforms[11].

In the paper, Src kinase activation is mandatory for MDA-9/syntenin-mediated activation of nuclear factor-κB, by H Boukerche, et al the authors state:

The scaffolding postsynaptic density-95/disks large/zonula occludens-1 (PDZ) domain-containing protein melanoma differentiation associated gene-9 (MDA-9)/syntenin is a tandem PDZ protein overexpressed in human melanoma, and breast and gastric cancer cells. MDA-9/syntenin affects cancer cell motility and invasion through distinct biochemical and signaling pathways, including focal adhesion kinase and p38 mitogen-activated protein kinase (MAPK), resulting in activation of the nuclear factor (NF)-κB pathway.

MDA-9/syntenin also promotes melanoma metastasis by activating c-Src, but how c-Src regulates NF-κB activation is unclear. Using a human melanoma model, we document that MDA-9/syntenin–c-Src interactions are positive regulators of NF-κB activation. Inhibition of c-Src by PP2 treatment, by blocking c-Src or mda-9/syntenin expression with small interfering RNA, or in c-Src (−/−) knockout cell lines, reduces NF-κB activation following overexpression of mda-9/syntenin or c-Src.

[11] http://www.ncbi.nlm.nih.gov/gene/6386

Deletion or point mutations of the PDZ binding motif preventing MDA-9/syntenin association with c-Src reveals that both PDZ domains, with PDZ2 being the dominant module, are required for activating downstream signaling pathways, including p38 MAPK and NF-κB. We also document that MDA-9/syntenin–c-Src complexes functionally cooperate with NF-κB to promote anchorage-independent growth, motility and invasion of melanoma cells. These findings underscore PDZ domains of MDA-9/syntenin as promising potential therapeutic targets for intervening in a decisive component of cancer progression, namely, metastatic tumor spread[12]*….*

(MDA-9 Acts as a PDZ domain-containing adapter protein. In adherens junctions, it couples syndecans to cytoskeletal proteins or signaling components. Seems to be required for the targeting of TGF-alpha to the cell surface in the secretory pathway. By virtue of its association with a large number of additional proteins, including class B ephrins, TGF-alpha, phosphotyrosine phosphatase, neurofaschin, neurexin, schwannomin/merlin, IL-5 receptor, various glutamate receptor subtypes, and the syndecan family of heparan sulfate proteoglycans, MDA9 has been implicated in diverse processes, including protein trafficking, activation of the transcription factor SOX4, cytoskeleton-membrane organization, and cell adhesion/migration….

(MDA-9) Its expression is induced by IFN-gamma in melanoma cells. Is believed to be involved in cancer metastasis. In melanoma, it promotes the metastatic phenotype by activating NFkB and focal adhesion kinase (FAK), which promotes induction of matrix metalloproteinase (MMP) and then migration and extracellular matrix invasion of melanoma cells. Syntenin is overexpressed and promotes cell migration in metastatic human breast and gastric cancer cell lines.

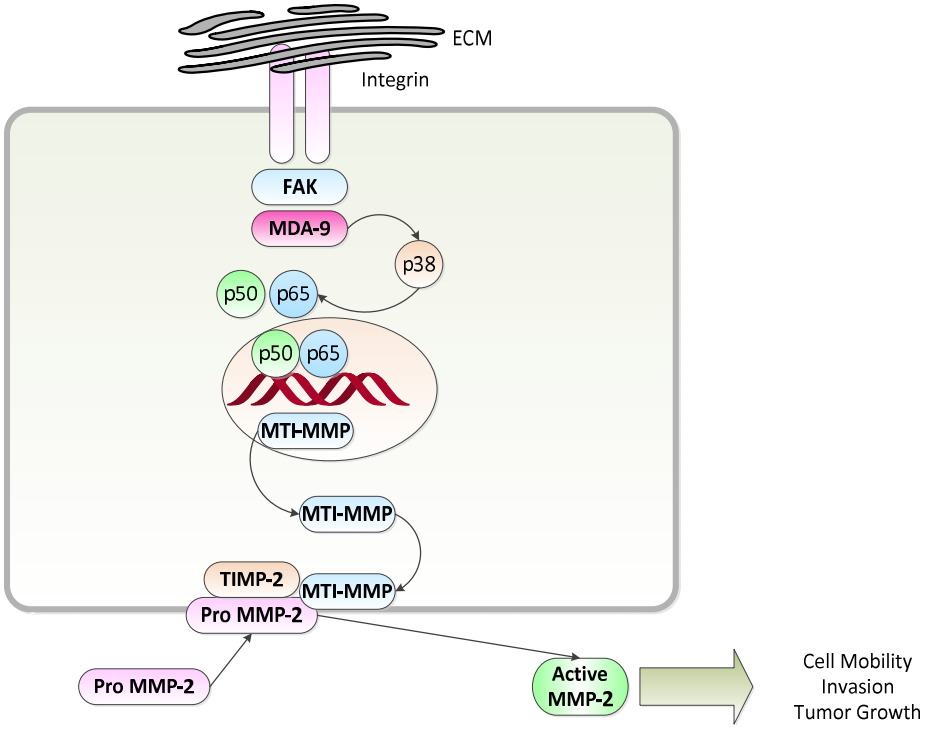
The gene product is also called by many other names, specifically:

1. MDA9
2. MDA-9
3. TGF alpha cytoplasmic domain interacting protein18
4. TACIP18
5. SYCL
6. Syntenin-1
7. Syndecan binding protein 1
8. SDCBP
9. Melanoma differentiation associated protein 9

From Das et al. we have the following modified figure[13]:

[12] http://www.nature.com/onc/journal/v29/n21/pdf/onc201065a.pdf

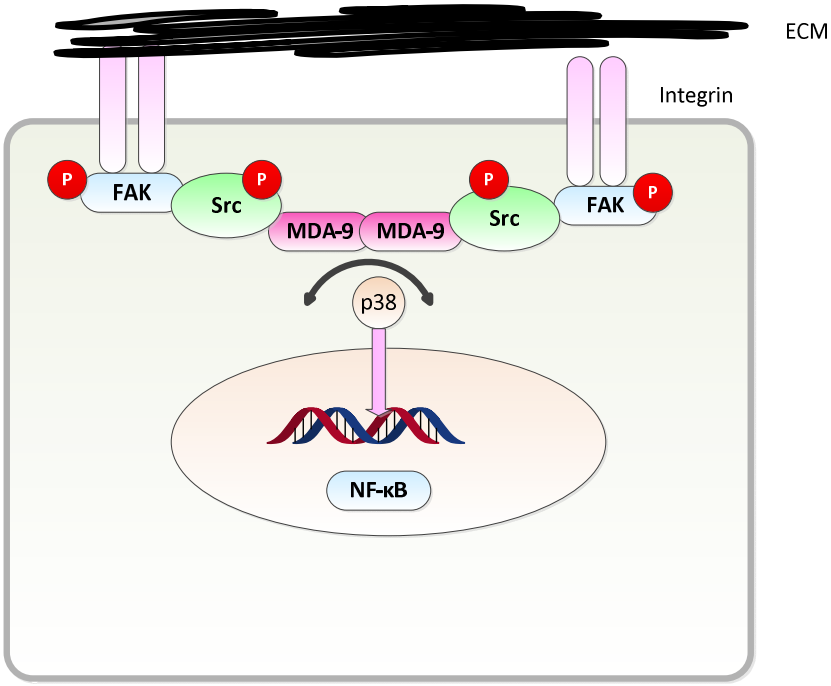
[13] http://www.bioscience.org/2012/v17/af/3911/fulltext.asp?bframe=figures.htm&doi=yes



Das et al state regarding the above pathway model:

Schematic diagram for mda-9/syntenin mediated NFκB activation. Upon interaction with ECM (fibronectin), MDA-9/syntenin activates the p38/MAPK by augmenting FAK phosphorylation. This results in degradation of IκBα and movement of p65 from the cytoplasm where interaction with p50 results in binding to target genes (MT1-MMP) resulting in enhanced production of MT1-MMP, which interacts with TIMP-2 activating pro-MMP-2 to produce active MMP-2. This product then enhances cell motility, invasion, and cancer cell growth. mda-9/Syntenin activates the NF-kB pathway.

The original Figure appears to be from Boukerche et al as shown with some mods below:



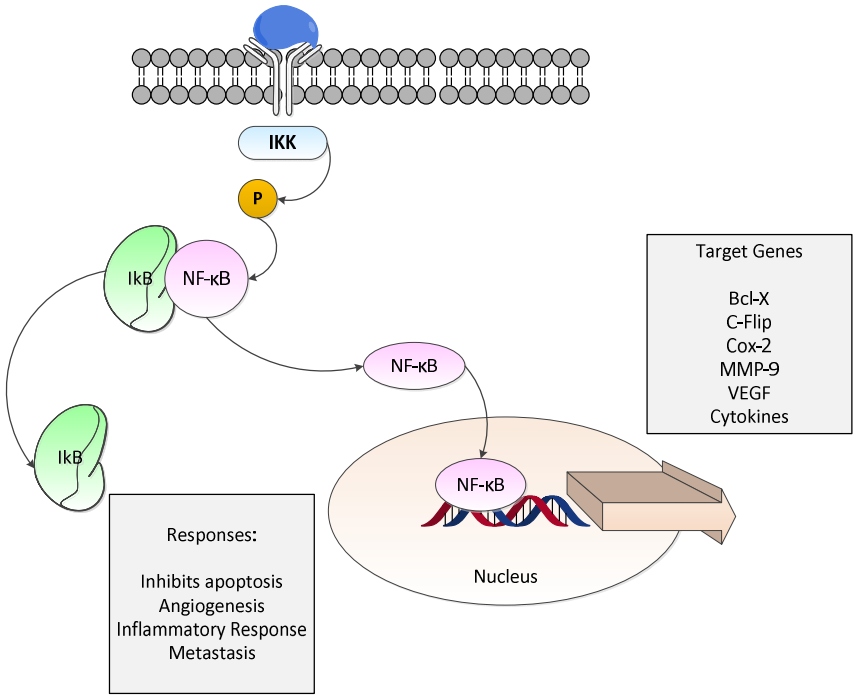
Note the differences. First the original shows multiple integrins and multiple FAK binding and in turn a binding of MDA-9 initiating the p38 pathway. Also note the explicit presence of NF-κB and its result of genes forcing mobility, invasion and metastasis. The authors state:

Hypothetical model of signal transduction pathways coordinately regulated by MDA-9/syntenin through its interaction with c-Src. MDA-9/ syntenin interaction with c-Src results in clustering of c-Src/FAK signaling complexes at high concentrations on the plasma membrane. The activated c-Src/FAK complexes activate the p38 MAPK/NF-κB pathways that regulate expression of genes involved in migration and invasion and Thus, play a crucial role in MDA-9/syntenin-mediated tumor progression.

The initiation of NF-κB is a significant factor since this transcription factor is what appears to be the instigator of the metastatic processes.

From Pecorino, p 220, we have again presented the details (as modified)[14]:

[14] Pecorino, Molecular Biology of Cancer, Oxford (New York) 2nd Ed, 2005.



The above graphic clearly demonstrates the movement of the transcription factor into the nucleus, from a bound state with IkB to an unbound and active state. The target genes indicated includes an MMP gene which again goes into the ECM.

As Sarkar et al state:

*Melanoma differentiation associated gene-9 (*mda-*9), also known as syntenin, is a PDZ domain–containing adapter protein that is involved in organization of protein complexes in the plasma membranes, regulation of B-cell development, intracellular trafficking and cell-surface targeting, synaptic transmission, and axonal outgrowth. Recent studies now define a seminal role for* mda-*9/sytenin in cancer metastasis*.

Thus, Sarkar who is part of Fisher's Lab at Virginia, have had a focus on Mda-9. They continue:

Adapter proteins play an essential role in modulating signal transduction from the extracellular environment to the intracellular milieu by virtue of their association with key regulatory molecules …

mda-9 was originally cloned as a gene differentially expressed in human melanoma cells reprogrammed to terminally differentiate by combination treatment with IFN-h and the protein kinase C activator mezerein … Analysis of the subcellular distribution of mda-9/syntenin revealed its localization at the areas of cell-cell contact in cells of epithelial origin in colocalization with F-actin, syndecan-1, E-cadherin, h-catenin, and a-catenin.

In fibroblasts, mda-9/ syntenin localizes to focal adhesions and in stress fibers. Overexpression of mda-9/syntenin in different cells induces the formation of plasma membrane structures,

including ruffles, lamellipodia, fine extensions, and neurite-like structures, showing its role in regulating the structure and function of the plasma membrane…

They continue:

The major characteristic of malignant tumor cells is their ability to invade foreign tissues and form metastatic foci at distant locations in the body. Such a process requires tumor cell attachment to various matrix proteins, degradation of the extracellular matrix (ECM) mainly by matrix metalloproteinases (MMP), followed by migration into the surrounding stroma by tumor cells…A model of progression of melanoma suggests that it begins by conversion of a normal melanocyte into a benign nevi, subsequent transformation into a radial and then a vertical growth phase primary melanoma, and finally evolution into a metastatic melanoma.

Finally, Sarkar et al outline the overall set of functions which MDA-9 is involved in. Specifically, they state:

1. ***Interleukin-5 signaling.*** *mda-9/syntenin interacts with interleukin- 5 (IL-5) receptor a and the transcription factor Sox4, Thus, mediating IL-5–induced Sox4 activation …*

2. ***Cell-surface trafficking.*** *Although mda-9/syntenin is located predominantly in the plasma membrane, it is also identified in the early secretory pathway such as the endoplasmic reticulum, intermediate compartment, and cis-Golgi, Thus, facilitating cellsurface trafficking of secreted molecules such as proTGF-a, an epidermal growth factor receptor ligand…*

3. ***mda-9/syntenin and ephrin signaling.*** *Ephrins and their cellsurface tyrosine kinase receptors are implicated in controlling axon guidance and fasciculation …*

4. ***Mediation of cohesiveness of epidermal stem cells.*** *In the basal layer of interfollicular epidermis the stem cells are clustered, a feature known as cohesiveness. These cells express high levels of Notch ligand D1, which is important for maintaining cohesiveness …*

5. ***Regulation of glutamate signaling.*** *The excitatory neurotransmitter glutamate interacts with its cognate receptors and regulates postsynaptic excitatory currents. Glutamate receptors interact with mda-9/syntenin, …*

6. ***Regulation of axon outgrowth.*** *Unc51.1 is a serine/threonine kinase that is important for neurite extension/parallel fiber formation in cerebellar granule neurons. mda-9/syntenin interacts with Unc51.1 and Rab5, a member of the Ras-like small GTPases that is a marker of early endosomes and is essential for endocytic membrane fusion and trafficking. …*

Boukerche et al (2005) stated:

Studies using an enhanced green fluorescent protein mda-9/ syntenin fusion protein showed that endogenous mda-9*/syntenin colocalized with the E-cadherin complex and syndecan-1 at adherens junctions as well as with focal adhesions and stress fibers at cell-substratum contact in*

fibroblastic and epithelial cells. These findings suggest that Mda-9*/syntenin might promote cytoskeletal organizational changes and intracellular signaling.*

The organization of these dissimilar focal contacts is complex but was shown not only to contain the appropriate integrin but also cytoskeletal proteins (vinculin, talin, and a*-actinin) as well as several cytoplasmic protein tyrosine kinases, including members of the src family and focal adhesion kinase (FAK). Despite extensive research documenting an ability of* mda-9*/syntenin to form multivalent interactions, little is known about the role of* Mda-9*/syntenin in cancer development.*

Boukerche et al (2008) state:

Prior studies confirm that Mda-9*/syntenin stimulates motility through pathways involving FAK, p38MAPK, and NF-κB, leading to secretion of MMP-2 (4, 9). However, despite these intriguing observations, it is not fully understood how* Mda-9*/syntenin orchestrates these signaling molecules to enhance cancer cell motility and metastasis. A complex network of protein-protein interactions characterizes the structural organization of focal adhesions, involving known signaling molecules that play functional roles in various cellular activities and other less well-defined pathways.*

We presently show that Mda-9*/syntenin interacts with c-Src through its PDZ domain and activates the c-Src/FAK signaling pathway to maximize tumor cell motility and anchorage-independent growth of melanoma cells.* Mda-9*/Syntenin levels directly correlate with increased c-Src activity in a human melanoma model that closely mimics the early events of metastasis in humans.*

In 2010 Boukerche et al report:

MDA-9/syntenin affects cancer cell motility and invasion through distinct biochemical and signaling pathways, including focal adhesion kinase and p38 mitogen-activated protein kinase (MAPK), resulting in activation of the nuclear factor (NF)-kappaB pathway.

MDA-9/syntenin also promotes melanoma metastasis by activating c-Src, but how c-Src regulates NF-kappaB activation is unclear. Using a human melanoma model, we document that MDA-9/syntenin-c-Src interactions are positive regulators of NF-kappaB activation. Inhibition of c-Src by PP2 treatment, by blocking c-Src or mda-9/syntenin expression with small interfering RNA, or in c-Src (-/-) knockout cell lines, reduces NF-kappaB activation following overexpression of mda-9/syntenin or c-Src.

Deletion or point mutations of the PDZ binding motif preventing MDA-9/syntenin association with c-Src reveals that both PDZ domains, with PDZ2 being the dominant module, are required for activating downstream signaling pathways, including p38 MAPK and NF-kappaB. We also document that MDA-9/syntenin-c-Src complexes functionally cooperate with NF-kappaB to promote anchorage-independent growth, motility and invasion of melanoma cells.

These findings underscore PDZ domains of MDA-9/syntenin as promising potential therapeutic targets for intervening in a decisive component of cancer progression, namely, metastatic tumor spread.

This set of papers from the Fisher Lab present several interesting connections between the ECM and the intra-cellular signaling paths. We have had prior arguments that one can develop models for metastasis by examining the cell as a target entity and then by modeling the environment, both the ECM and surrounding cells as influences on the target cell. In this work we can expand it to include ECM factors in some detail.

The suggested control of other pathway elements, beyond just the B-RAF control that we now have may be proven productive. Notwithstanding it does establish a research path that is based upon established cell dynamics.

4.2 MACROPHAGES

We return to the macrophages. From Salinas et al further delineate the M1 and M2 distinctions:

Macrophages can be divided schematically into two main classes in line with the Th1/Th2 dichotomy.

1. M1 macrophages (classically activated cells) originate upon encounter with IFN[15]-and microbial stimuli such as LPS and are characterized by IL-12high and IL-23 production and consequent activation of polarized type I T cell response, cytotoxic activity against phagocytozed microorganisms and neoplastic cells, expression of high levels of ROI, and good capability as APCs.

In general, M1 macrophages act as soldiers: they defend the host from viral and microbial infections, fight against tumors, produce high amounts of inflammatory cytokines, and activate the immune response.

2. On the other hand, distinct types of M2 cells differentiate when monocytes are stimulated with IL-4 and IL-13 (M2a), with immune complexes/TLR ligands (M2b), or with IL-10 and glucocorticoids (M2c).

Hallmarks of M2 macrophages are IL-10 high IL-12 low IL-1ra high IL-1 decoyRhigh production, CCL17 and CCL22 secretion, high expression of mannose, scavenger and galactose-type receptors, poor antigen-presenting capability and wound-healing promotion.

Further, M2 express specific change in some metabolic pathways: arginine metabolism is oriented toward the production of ornitine and polyamine instead of citrulline and NO.

[15] From Abbas et al, *IFN-γ activates macrophages to kill phagocytosed microbes. Macrophage activation resulting in increased microbicidal activity is called classical macrophage activation, to be contrasted with an alternative activation pathway that is induced by Th2 cytokines; these types of macrophage activation are described in more detail later.*

M2 cells are workers of the host: they promote scavenging of debris, angiogenesis, remodeling and repair of wounded/damaged tissues. Of note, M2 cells control the inflammatory response by down-regulating M1-mediated functions.

In addition, M2 macrophages are competent effector cells against parasitic infections. The loss of equilibrium of M1 and M2 cell number may lead to pathological events: an M1 excess could induce chronic inflammatory diseases, whereas an uncontrolled number of M2 could promote severe immune suppression.

As Quaranta and Schmid note:

*Macrophages originate from t**hree different developmental pathways**.*

*All tissue embryonic macrophages derive from macrophage precursors in the yolk sac and fetal liver. During adulthood, fetal macrophages are replaced gradually by macrophages derived from bone marrow **hematopoietic stem cell (HSCs)**.*

*Some types of tissue resident macrophages, including bone osteoclasts, **epidermal Langerhans** cells, lung alveolar macrophages, microglia and **liver Kupffer** cells develop from **embryonic macrophages** and persist in adult tissues independently of replenishment by Ly6Chigh monocytes originated from HSCs during adulthood.*

*Instead, other types of tissue macrophages such as intestine, dermis, heart and pancreas macrophages u**ndergo a continuous turnover in adulthood by recruitment of circulating monocytes which differentiate into macrophages upon tissue infiltration**.*

Infiltrating monocytes derived from HSCs are also the main source of macrophage replenishment into inflamed and remodeling tissues, and this process is driven by cytokines and chemokines, such as C-C motif chemokine ligand (CCL) 2, CCL5 and macrophage colony stimulating factor-1 (CSF-1).

*There are different markers to identify monocyte-macrophages diversity in human and mouse. In human, c**irculating monocytes, which originate from the bone marrow, can be classified in two subsets**:*

(i) CD14+ CD16neg 'inflammatory' or 'classical' and

(ii) CD14+ CD16+ 'patrolling' or 'non-classical' monocytes.

In the same way, mouse 'inflammatory' monocytes are classified as CD11b+ Ly6Chigh CCR2high CX3CR1low, in contrast 'patrolling' monocytes are CD11b+ Ly6Glow CCR2low CX3CR1high.

***Patrolling monocytes monitor the microvasculature under steady-state** conditions and rarely extravasate into tissue. However, they can rapidly accumulate in lung metastatic tissue and*

inhibit cancer cell seeding and growth by exerting anti-tumour functions through recruitment of NKs. Macrophages are a population of heterogeneous and plastic cells.

Once resident in tissues, macrophages acquire a distinctive phenotype in response to different signals present in the immediate microenvironment. Environmental stimuli, like IFN or microbial products, like the lipopolysaccharide

From Ruffell and Coussens:

Macrophages produce an array of cytokines, chemokines, polypeptide growth factors, hormones, matrix-remodeling proteases, and metabolites, many of which possess tumor-promoting activities. A caveat to some of these reported activities is that many findings originate from cell culture studies utilizing neoplastic myeloid cell lines or bone marrow-derived macrophages and, therefore, cannot account for the complex milieu of polarization signals to which macrophages would be exposed in vivo. This includes the aforementioned CSF-1 and CCL2, prostaglandin E2 (PGE2), and damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 protein (HMGB1), extracellular ATP, and degraded extracellular matrix components …

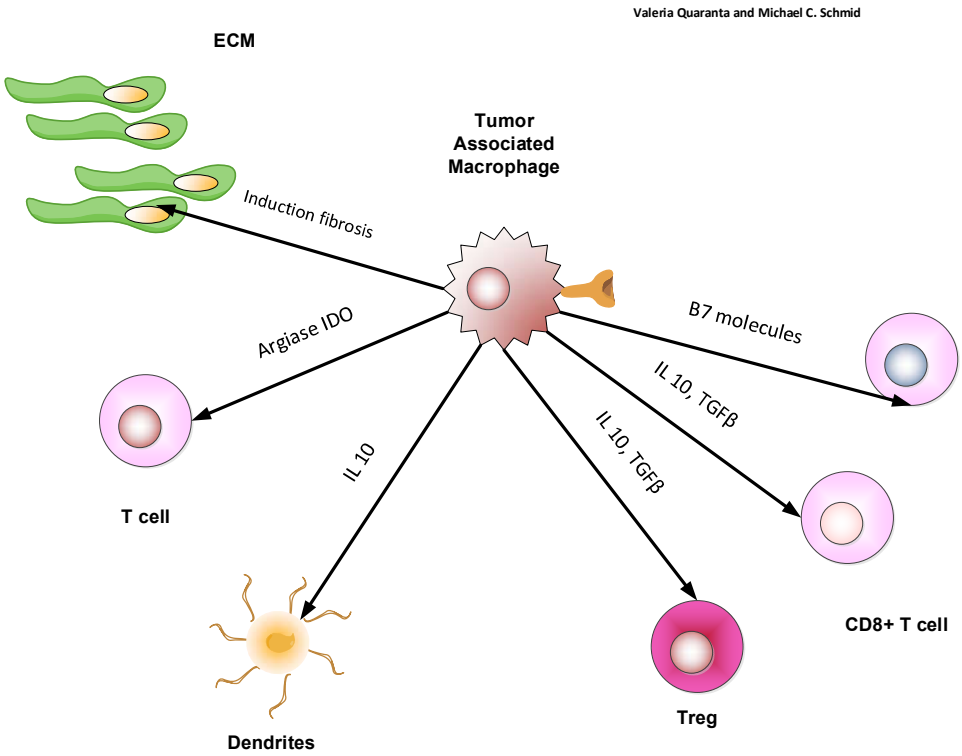
Macrophages are well described regulators of tumor angiogenesis, with supporting evidence derived from both clinical and experimental studies in which much of their capability is associated with vascular endothelial growth factor (VEGF) signaling. This includes macrophage production of VEGF-A, production of VEGF homologs such as placental growth factor, enhancement of VEGF-A bioavailability through matrix metalloproteinase (MMP)-9 activity, and induction of VEGF-A production by endothelial cells via WNT7B expression. VEGF-A drives the formation of abnormal vasculature in tumors, consisting of excessive branching, dead-end vessels, and vessel leakiness, that, together, impact tumor hemodynamics and drug delivery.

VEGF antagonists induce vascular normalization, and several studies have reported increased uptake of chemotherapeutics associated with this process, likely because of reduced vessel leakiness and interstitial fluid pressure. Although macrophages are not necessarily a dominant source of VEGF-A in all tumor tissues, specific deletion of VEGF-A in macrophages via lysozyme Mpromoter-driven Cre recombinase revealed their role in driving abnormal vascular phenotypes in tumors.

Importantly, similar to the use of VEGF antagonists, tumors in these mice were more sensitive to chemotherapy, although, unexpectedly, they also grew at a faster rate because of improved tissue perfusion and reduced hypoxia in the absence of therapeutic intervention…

4.2.1 Tumor Associated Macrophages

Let us begin with macrophages. To the beginning student of the immune system one often sees the macrophage as that wandering cell that sense invaders and then sends out signals as to their presence. In a simple sense this is the case. But then again as with all immune system elements it is always more than that.



Grivennikov et al note:

The most frequently found immune cells within the tumor microenvironment are tumor-associated macrophages (TAMs) and T cells. TAMs mostly promote tumor growth and may be obligatory for angiogenesis, invasion, and metastasis, and high TAM content generally correlates with poor prognosis.

As DeVita et al have noted[16]:

For example, tumor-associated macrophages (TAMs) can comprise a large proportion of tumor bulk. TAMs are often found at points of basement membrane breakdown and at the invasive front. By producing uPA, MMP7, and MMP9, TAMs help tumors degrade extracellular proteins.

The numerous growth factors that TAMs produce:

FGF, fibroblast growth factor

EGF, epidermal growth factor receptor ligands, and

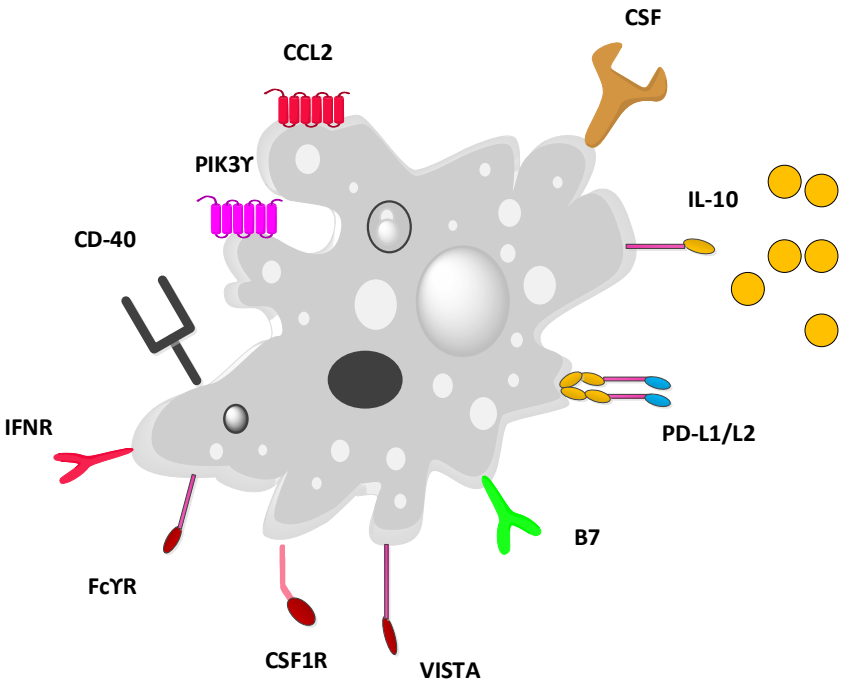
PDGF, platelet derived growth factor, stimulates tumor cell growth and motility.

[16] DeVita et al p 124

As in normal wound healing, these growth factors secreted by the TAMs or the tumors themselves activate fibroblasts.

These carcinoma-associated fibroblasts (CAFs) promote primary tumor growth by secreting stromal cell-derived factor 1 (SDF-1 or CXCL12), the ligand for CXCR4 on tumor cells. Angiogenesis is also aided by the action of CAFs through recruitment of endothelial progenitor cells by CXCL12 ***and by the action of TAMs that are recruited to areas of hypoxia to produce VEGF****. To ensure the loyalty of TAMs in promoting tumor growth, the tumor microenvironment can contain immunomodulatory factors like* ***TGF-β****, cyclooxygenase-2 (COX2),* ***CSF-1*** *(macrophage growth factor, colony-stimulating factor-1), IL-10, and IL-6, which inhibits maturation of dendritic cells and promotes TAMs that are immunosuppressed*

The TAM appears as below in terms of its receptors.



We shall examine these surface proteins in some detail as they apply to the development of a malignancy. Now as DiNardo and Ruffel note:

The presence of tumour-associated macrophages (TAMs) is generally associated with a poor prognosis in solid tumours. This has been shown in studies performed on individual tumour types using traditional immunohistochemistry techniques to quantify cellular density and in more recent analyses that infer the presence of macrophages across malignancies using gene expression profiles. These findings are consistent with the established role of macrophages in promoting multiple aspects of tumorigenesis in experimental models, from initiation through to angiogenesis and systemic dissemination.

Most relevant for patients, TAMs are known to suppress responses to standard-of-care therapeutics, including chemotherapy, irradiation and angiogenic inhibitors. Although this includes direct regulation of survival and cell death pathways in tumour cells in vivo modelling indicates that improved efficacy following macrophage depletion is often dependent upon enhanced recruitment or function of cytotoxic CD8+ T cells.

Perhaps not surprisingly, macrophage antagonists demonstrate combinatorial efficacy when combined with immunotherapy, including checkpoint blockade. Clinical trials examining these combinations are now ongoing. In this Review, we discuss how macrophages are induced into becoming immunosuppressive, the mechanisms by which they suppress antitumour immunity and how this information is being utilized to develop therapeutics and design clinical trials.

From Wilke et al in Curiel we have:

TAMs (tumor associated macrophages) form the major APC subset (by number) in solid human epithelial cancers. Several years ago, our group discovered that both tumor cells and microenvironmental macrophages in ovarian cancer expressed CCL22, a chemokine instrumental in attracting Tregs to the tumor environment.

Interestingly, because the presence of Tregs predicts poorer survival and is associated with a high death hazard in ovarian cancer patients, TAMs may contribute to their prognoses. Indeed, we subsequently demonstrated that although they are highly B7-H4 positive, ovarian cancer cells do not directly mediate antitumor T cell suppression. However, B7-H4+ macrophages from the human ovarian tumor microenvironment are powerful suppressors of tumor-associated antigen-specific T cell immunity. B7-H4 blockade restored the stimulatory capacity of macrophages and mediated ovarian tumor regression in vivo in NOD/SCID mice. Both IL-10 and IL-6, often found in high concentrations in the tumor environment, can induce B7-H4 expression on macrophages.

Contrastingly, two cytokines minimally expressed in the same environment—GM-CSF and IL-4—inhibit B7-H4 expression. Interestingly, forced expression of B7-H4 in macrophages from healthy donors conferred a suppressive phenotype on the cells. As for the prognostic significance of B7-H4+ macrophages in ovarian cancer, we documented an inverse relationship between the intensity of B7-H4 expression on macrophages and patient survival. Importantly, Tregs, typically predictors of poor prognoses in cancer patients, could induce B7-H4 expression on myeloid APCs (including macrophages) and were positively associated with B7-H4+ macrophage presence in ovarian tumors.

A later observation of Wan and colleagues showed that the mean density of TAMs is significantly higher in ovarian cancer than in benign ovarian lesions and that the average 5-year survival rate in patients with low densities of TAM was significantly higher than in patients with larger TAM populations, agreeing well with our observations. Multivariate analysis demonstrated that TAM infiltration status serves as an independent negative predictor for overall survival of patients with ovarian cancer. The presence of CCL17+ or CCL22+ cells in CD14+ monocytes and macrophages within gastric tumors correlated directly with Treg cell presence. Tregs were also shown to migrate toward CCL17 and CCL22

Kundu and Surh note:

Tumor-associated macrophages, mast cells and neutrophils play an important role in tumor angiogenesis by secreting VEGF, IL-8, TNFa, MMPs and other factors that increase vascular permeability.

Thus, chronic inflammation-driven tumor angiogenesis and a sustained 'inflammation-cancer-inflammation' loop proves Dvorak's early proposition that tumors are wounds that never heal. The role of various proinflammatory mediators in tumor angiogenesis will be discussed further.

Poh and Ernst note a more differentiated characterization of M1 and M2, separating M2 into four subsets as follows:

Tumor-associated macrophage heterogeneity is not only dependent on the nature of their monocytic precursor, but also on their functional diversity. To coordinate complex processes to promote immunity, while also minimizing damage to tissues where these responses occur, macrophages can reversibly alter their endotype in response to environmental cues.

These environmental cues include stimuli derived from pathogens, parenchymal, and immune cells, as well as the extracellular matrix. Similar to the Th1/Th2 T-cell dichotomy, macrophages may be broadly classified into two groups, referred to as:

(i) ***"classically activated M1" (CAM) or***

(ii) ***"alternatively activated M2" (AAM) endotypes.***

Much our understanding of macrophage polarization has relied on ***in vitro techniques****, whereby macrophages are stimulated with M1- or M2-polarizing signals.*

(i) ***For M1 this typically involves stimulation with IFNγ or lipopolysaccharide (LPS),***

(ii) ***while M2 polarization usually involves stimulation with IL4 or IL13.***

Changes in gene expression, cell-surface markers and signaling pathways have subsequently been used to distinguish the various activation states, and the contribution of some of these factors in mediating CAM/AAM characteristics has been validated in genetically engineered mouse models.

However, given the heterogeneity of tissues, macrophage polarization should be regarded as a complex process that occurs over a continuum. The current classification of CAM or M1 macrophages is in part based on their response to stimulation with bacterial LPS, TNFα, and/or IFNγ. TNFα is produced by antigen presenting cells upon recognition of pathogenic signals, while IFNγ is produced by innate and adaptive immune cells such as natural killer (NK) and Th1 cells. Once activated, CAMs secrete pro-inflammatory cytokines (IL1, IL6, and TNFα) and

effector molecules (including reactive nitrogen intermediates) and express chemokines such as CXCL9 and CXCL0.

These molecules exert and amplify antimicrobial and tumoricidal activities alongside increased Th1 adaptive immune responses through enhanced antigen presentation. Because these cytokines play an important role in immune defense, their inappropriate release can result in chronic inflammation and extensive tissue damage.

***Alternatively activated M2 macrophages are broadly characterized by their anti-inflammatory and wound-healing endotype**. While these functional outputs are important for the maintenance of tissue homeostasis, aberrant AAM activation can trigger allergic reactions, promote tumor growth, and delay immune responses toward pathogens.*

Among the most important activators of AAMs are IL4, IL10, and IL13; however, several other stimuli and signaling pathways can also induce AAM polarization.

Thus, AAMs can be further divided into M2a, M2b, M2c, and M2d. The M2a subtype is stimulated in response to IL4, IL13, as well as fungal and helminth infections.

M2a macrophages express high levels of mannose receptor (CD206) and secrete large amounts of pro-fibrotic factors including fibronectin, insulin-like growth factor and TGFβ, which are all involved in wound healing and tissue repair.

***M2b macrophages are stimulated by immune complexes and bacterial LPS and exhibit upregulated expression of CD206 and the MER receptor tyrosine kinase.** They primarily produce IL10, IL1β, IL6, and TNFα, which exert anti-inflammatory effects.*

***M2c macrophages** are activated by IL10, TGFβ, and glucocorticoids and are also generally thought to be anti-inflammatory in nature. Finally, differentiation of*

***M2d macrophages** occurs in response to co-stimulation with **TLR ligands and adenosine.** M2d macrophages express low levels of CD206 but are high producers of **IL10 and VEGF**. In light of these findings, it is now appreciated that the "AAM" terminology encompasses a functionally diverse group of macrophages that share the functional outputs of tumor progression by stimulating immunosuppression and angiogenesis.*

We summarize the above in the following table.

Type	*Activated by*	*Produce*
M1	*stimulation with IFNγ or lipopolysaccharide (LPS)*	
M2a	*stimulation with IL4 or IL13*	*mannose receptor (CD206) and secrete large amounts of pro-fibrotic factors including fibronectin, insulin-like growth factor and TGFβ,*
M2b	*by immune complexes and bacterial LPS*	*upregulated expression of CD206 and the MER receptor tyrosine kinase.*
M2c	*activated by IL10, TGFβ, and glucocorticoids*	
M2d	*co-stimulation with TLR ligands and adenosine*	*CD206 but are high producers of IL10 and VEGF.*

From Laviron and Boissonnas we have an interesting reconfiguration of this M1 and M2 fabric. They authors present a somewhat alternative view as follows:

Tumor-associated macrophages (TAM) represent a major component of the tumor microenvironment (TME) that has been extensively studied in the past decades. They play a major role in tumor growth, metastatic dissemination, and therapy failure. Countless reports have described that TAMs can promote angiogenesis, inhibit the anti-tumor immune response, in particular T-cell-mediated cytotoxicity, support tumor growth, and secrete different factors involved in extracellular matrix (ECM) remodeling thus facilitating tumor cell motility and intravasation. High TAM infiltration is generally correlated with poor outcomes in several types of cancer, such as breast, ovarian, and lung cancer.

However, in some indications TAM can be associated with enhanced anti-tumor immunity. Although macrophages were originally described as arising exclusively from circulating monocyte precursors, it was shown in the recent years that several organs harbor embryonic-derived populations of ***resident macrophages (ResMac)*** *that maintain and self-renew throughout adulthood.*

This new concept ***challenges the dogma of TAM origin and questions their relative function. TAM subsets were originally classified as tumoricidal vs. tumor-promoting, often referred as M1/M2 macrophages,*** *based on the expression of specific markers. However, the wide diversity of TAM cannot be covered by this nomenclature and many subsets express overlapping markers of the M1/M2 polarization.*

Whether TAM heterogeneity originates from their high plasticity or rather from independent specific lineages giving rise to multiple populations is still unclear. Although cellular ontogeny can recapitulate parts of the heterogeneity, it appears that environmental cues are also major

determinants in cell education. Macrophage diversity would then be the result not only of ontogeny but also of niche- specific signaling events of tumor immunity.

One can thus wonder whether the origin of TAM dictates their role in tumor development and is associated with various functions. This represent a key issue for anti-cancer therapies as these subsets might be differentially targeted regarding their role in tumor development. …

Although the precise origin of ResMac is still under debate, fate-mapping models highlighted a differential origin of tissue macrophages deriving either from an embryonic precursor (yolk sac, fetal liver) or a monocyte precursor from adult hematopoiesis origin.

***These precursors seed the tissues in different waves during development and adulthood giving rise to different ResMac**. The dynamics of these waves vary between organs, age, and macrophage subsets.*

In some organs, such as the brain, the lung and the liver,

*(i) some **embryonic-derived ResMac (named here EmD-ResMac)** maintain by self-renewal in adults whereas in the gut, the skin, the heart, and the pancreas*

*(ii) most subsets are progressively replaced through the dierentiation of monocyte precursors from adult hematopoiesis into **monocyte-derived ResMac (named here MoD-ResMac**) with different turnover rates.*

The ability of newly recruited macrophages to self-maintain in the tissue and become a ResMac per se is proposed to be tightly regulated by space availability and competition for growth factors in the niche. This turnover appears to be variable among subsets in a given organ and could be induced by exposure to homeostatic environmental cues (e.g., mechanical, metabolic) specific of distinct sub-tissular regions.

In the gut, long-lived macrophages with precise sub-tissular localization are key regulators of physiological functions. In the lungs, alveolar macrophages (AM) originate almost exclusively from yolk-sac derived macrophages and self-maintain throughout adulthood, whereas lung interstitial macrophages follow a more complex regulation, unveiling further heterogeneity in this subset. While some of these interstitial macrophages have an embryonic origin, others differentiate from distinct monocyte precursors according to the sub-tissular niche they colonize, thus becoming the dominant population during adulthood. …

The common characterization of TAM subsets relies on the M1/M2 polarization model induced by different in vitro stimuli. This model rapidly finds limitation in complex environments (in vivo) in which M1 and M2 stimuli can be present and generate very dynamic microanatomical niches.

Tumors should be considered as an evolving tissue in which space availability and growth factors expression are changing over time and where inflammatory signals are generated by the loss of tissue integrity and immune cell infiltration.

It is thus not surprising to find a wide range of activation profiles in the TME. No typical M1/M2-associated marker defined one or the other TAM subset in lung unveiling heterogeneity among each subset.

No direct link between TAM origin and the commonly described pro- or anti-tumor profile could be achieved in this study. One could expect that macrophage ontogeny and their anatomic localization define specific niches dictating their polarization toward a specific phenotype and function.

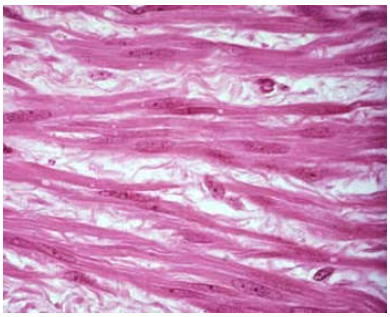
Thus, one may conclude that the TAMs are of varying types activating and being activated in a multiplicity of ways.

4.3 FIBROBLASTS

Fibroblasts are common cells that generally do not form any specific functioning collection of cells. The fibroblast is resident in the stroma of most organs.

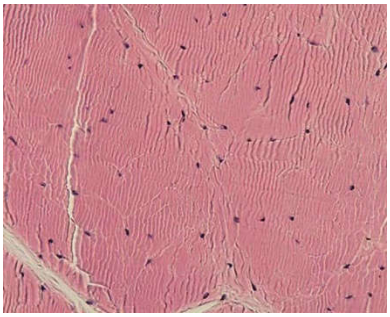
4.3.1 Histology

We start by examining the fibroblasts histologically. Fibroblasts seem to be almost universal and part of the vast connective matrix. An example is shown below[17]:

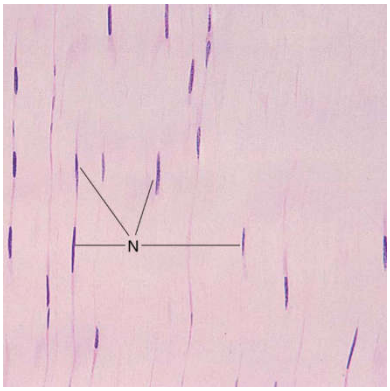


The following are from Gartner and Hiatt:

[17] http://www.meddean.luc.edu/lumen/MedEd/Histo/frames/h_frame7.html



and the following is another example from the same source.



In both of the above cases the fibroblasts are elongated, prominent nuclei and somewhat clear protoplasm. All of the above fibroblasts and long tear shaped cells with prominent nuclei. They generally are unorganized and have a clear cytoplasm.

4.3.2 Fibroblast Functions

From NCBI we have[18]:

The fibroblast is one of the most abundant cell types present in the stroma. It has a variety of functions and composes the basic framework for tissues and organs. Under homeostasis, this cell is responsible for maintaining the extracellular matrix (ECM). During stress, fibroblasts adapt to their environment and have the ability to respond and send local signals. In times of injury, the fibroblast can transform phenotypes and synthesize the building blocks necessary to replace wounded tissue. During pathologic states, the extracellular matrix gets generated in excessive quantities, and collagen is deposited in a dysregulated manner often causing irreversible organ dysfunction or disfiguring appearance….

Fibroblasts are the most common cell type represented in connective tissue. These cells produce a diverse group of products including collagen type I, III, and IV, proteoglycans, fibronectin, laminins, glycosaminoglycans, metalloproteinases, and even prostaglandins. In the adult body,

[18] https://www.ncbi.nlm.nih.gov/books/NBK541065/

fibroblasts remain in a quiescent form until stimuli activate protein synthesis and contractile mechanisms.

These cells synthesize reorganize the ECM found in the skin, lung, heart, kidney, liver, eye, and other organs. The ECM is in constant communication with the surrounding cells as fibroblasts can secrete and respond to both autocrine and paracrine signals. Matrix reorganization occurs through a process of degradation and crosslinking enzymes, produced by fibroblasts, that are activated and regulated by pro-inflammatory cytokines and growth factors. Transcription growth factor-alpha and beta (TGF-A and TGF-B), platelet-derived growth factor (PDGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), epidermal growth factor (EGF), and tumor necrosis factor (TNF) all have implications in fibroblast regulation.

The relationship with the ECM is an important factor especially when we examine its role in various cancers. They continue:

Fibroblasts are a diverse group of cells. Within one organ system, there can be a great variety of functions. Within the integument, dermal fibroblasts in different locations have separate roles. The superficially located lineage involves the formation of the hair follicle and is responsible for reepithelization during wound healing; the deeper lineage is responsible for ECM generation.

Fibroblasts are known for their plasticity; adipocytes, pericytes, endothelial and epithelial cells, otherwise known as terminally differentiated cells, can de-differentiate into fibroblasts.

Stimulation of fibroblasts further increases susceptibility to epigenetic modifications. The ability of fibroblasts to transform is partly due to the variety of cell-surface adhesion receptors (integrins, syndecans, cadherins) that facilitate the communication of fibroblasts with their surroundings. One of these well-described fibroblast transformations is the transformation of fibroblast into the myofibroblast.

Myofibroblasts are present in both healthy and pathologic tissues and contain features of fibroblasts and smooth muscle cells. These cells work in conjunction with vascular endothelial cells to form granulation tissue during times of wound healing.

In the following, many of the cancer related involvements of fibroblasts will focus on the transitioned myofibroblast.

4.3.3 Scars and Markers

Identifying fibroblasts are generally done histologically by visible inspection but they also can be further classified by surface markers. Now this is discussed in Ziani et al who note:

Fibroblasts are spindle-shaped, non-epithelial (cytokeratin negative, E-cadherin negative), non-endothelial (CD31 negative) and non-immune (CD45 negative) cells of a mesenchymal lineage origin (vimentin+). In normal tissue, fibroblasts are usually considered as resting/ quiescent cells with negligible metabolic and transcriptional activities, but with the ability to respond to growth factors to become activated.

This is an exceptionally short and clear description. The fibroblasts are cells somewhat on their own and are interstitial to organ focused cells. The lack of E cadherin allows them to have substantial mobility.

During this activation process, fibroblasts exhibit contractile activity, exert physical forces to modify tissue architecture, acquire proliferation and migration properties and become transcriptionally active leading to the secretion of several factors (cytokines, chemokines, etc.) and ECM components.

The ability of resting fibroblasts to become activated was first observed in the context of wound healing and subsequently in pathologic conditions such as acute or chronic inflammation or tissue fibrosis (a chronic wound healing response).

***This chronic tissue repair response also occurs in the context of cancer, considered as a "wound that never heals"**.*

This concept of wound healing is a significant driver of understanding how fibroblasts play such a role in cancers. Wound healing is the process in humans of repairing damaged organs and in turn cells. It is a tissue repair attempt, albeit one poorly accomplished, yet its ultimate protective result allows and facilitates a malignant growth.

Indeed, emergence and/or accumulation of cancer cells in a given tissue represent a tissue injury, imitating a chronic wound healing response toward the tumor cells, also known as tumor fibrosis or desmoplastic reaction.

Consequently, major players in tumor fibrotic microenvironment include activated fibroblasts, termed cancer-associated fibroblasts (CAFs), which represent one of the most abundant stromal cell types of several carcinomas including breast, prostate, pancreatic, esophageal, and colon cancers while CAFs are less abundant, but still present, in other neoplasias including ovarian, melanoma, or renal tumors. For example, in pancreatic cancer, 60–70% of the tumor tissue is composed of a desmoplastic stroma characterized by extensive collagen deposition and activated CAFs.

Now it is the CAF that we will focus upon. However, the key issue to note is that the fibroblasts play a significant role in wound repair. As Kumar et al note:

*Several cell types proliferate during tissue repair. These include the remnants of the injured tissue (which attempt to restore normal structure), vascular endothelial cells (to create new vessels that provide the nutrients needed for the repair process), and **fibroblasts (the source of the fibrous tissue that forms the scar to fill defects that cannot be corrected by regeneration).***

The ability of tissues to repair themselves is determined, in part, by their intrinsic proliferative capacity. In some tissues (sometimes called labile tissues), cells are constantly being lost and must be continually replaced by new cells that are derived from tissue stem cells and rapidly proliferating immature progenitors.

These types of tissues include hematopoietic cells in the bone marrow and many surface epithelia, such as the basal layers of the squamous epithelia of the skin, oral cavity, vagina, and cervix; the cuboidal epithelia of the ducts draining exocrine organs (e.g., salivary glands, pancreas, biliary tract); the columnar epithelium of the gastrointestinal tract, uterus, and fallopian tubes; and the transitional epithelium of the urinary tract. These tissues can readily regenerate after injury as long as the pool of stem cells is preserved.

Other tissues (called stable tissues) are made up of cells that are normally in the G0 stage of the cell cycle and hence not proliferating, but they are capable of dividing in response to injury or loss of tissue mass. These tissues include the parenchyma of most solid organs, such as liver, kidney, and pancreas. ***Endothelial cells, fibroblasts, and smooth muscle cells are also normally quiescent but can proliferate in response to growth factors, a reaction that is particularly important in wound healing.***

Now they continue on the process of developing a scar, or scar tissue as follows:

1. Within minutes after injury, a hemostatic plug comprised of platelets is formed, which stops bleeding and provides a scaffold for infiltrating inflammatory cells.

2. Inflammation. This step is comprised of the typical acute and chronic inflammatory responses. Breakdown products of complement activation, chemokines released from activated platelets, and other mediators produced at the site of injury function as chemotactic agents to recruit neutrophils and then monocytes during the next 6 to 48 hours. As described earlier, these inflammatory cells eliminate the offending agents, such as microbes that may have entered through the wound, and clear the debris. Macrophages are the central cellular players in the repair process—M1 macrophages clear microbes and necrotic tissue and promote inflammation in a positive feedback loop, and M2 macrophages produce growth factors that stimulate the proliferation of many cell types in the next stage of repair. As the injurious agents and necrotic cells are cleared, the inflammation resolves; how this inflammatory flame is extinguished in most situations of injury is still not well defined.

3. Cell proliferation. In the next stage, which takes up to 10 days, several cell types, including epithelial cells, endothelial and other vascular cells, and fibroblasts, proliferate and migrate to close the now-clean wound. Each cell type serves unique functions.

a. *Epithelial cells respond to locally produced growth factors and migrate over the wound to cover it.*

b. *Endothelial and other vascular cells proliferate to form new blood vessels, a process known as angiogenesis. Because of the importance of this process in physiologic host responses and in many pathologic conditions, it is described in more detail later.*

c. ***Fibroblasts proliferate and migrate into the site of injury and lay down collagen fibers that form the scar.***

d. The combination of proliferating fibroblasts, loose connective tissue, new blood vessels and scattered chronic inflammatory cells, forms a type of tissue that is unique to healing wounds and is called granulation tissue. This term derives from its pink, soft, granular gross appearance, such as that seen beneath the scab of a skin wound.

4. Remodeling. The connective tissue that has been deposited by fibroblasts is reorganized to produce the stable fibrous scar. This process begins 2 to 3 weeks after injury and may continue for months or years.

Now as we noted earlier, this process seems to occur with the introduction of malignant cells as well. Unlike a normal benign scar, however, a malignant scar or tumor, uses the same elements but it does so in a manner to protect itself. It uses the fibroblasts as a tool for protection.

4.3.4 FGF

Growth factors are many in number and are often key players in the proliferation of cancers. Fibroblast growth factors, FGF, are broadly functioning growth factors. They obtained their names from their initial discovery on fibroblasts but they are more common than just that. They often activate a variety of kinase pathways in cells and play a significant role in multiple malignancies.

As Yun et al have noted regarding the historical linkage to fibroblasts as follows:

Fibroblast growth factor (FGF) is a representative growth factor which has shown the potential effects on the repair and regeneration of tissues.

It was originally identified as a protein capable of promoting fibroblast proliferation and is now known to comprise 22 members*.*

FGFs exert multiple functions through the binding into and activation of fibroblast growth factor receptors (FGFRs), and the main signaling through the stimulation of FGFRs is the RAS/MAP kinase pathway. With their potential biological functions, FGFs have been utilized for the regeneration of damaged tissues, including skin, blood vessel, muscle, adipose, tendon/ligament, cartilage, bone, tooth, and nerve.

Then, the prospective source of FGF for the tissue regeneration is used with recombinant human FGF family. In fact, many previous studies administered the FGFs directly to the wound sites, like other growth factors. However, free-FGFs are readily degradable in vivo, leading to loss of biological activity and functions. To gain satisfactory performance, FGFs are adsorbed onto or encapsulated within materials to secure biological activity in a sustained and controllable manner. Although many types of materials have been developed to carry FGFs and elicit their therapeutic efficacy in vitro and in vivo, more sustained, controlled, and targeted delivering system still remain a challenge

Thus, FGFR obtain their name in an historical manner based upon the vehicle in which they were first identified yet have a wide range of functionality.

4.3.5 FGF Functions

We now want to examine some of the functionality of the FGF.As Teishima et al have recently noticed in a discussion on prostate cancer:

Fibroblast growth factors (FGFs) and FGF receptors (FGFRs) play an important role in the maintenance of tissue homeostasis and the development and differentiation of prostate tissue through epithelial-stromal interactions. Aberrations of this signaling are linked to the development and progression of prostate cancer (PCa). The FGF family includes two subfamilies, paracrine FGFs and endocrine FGFs.

***Paracrine FGFs directly bind the extracellular domain of FGFRs and act as a growth factor through the activation of tyrosine kinase signaling**.*

Endocrine FGFs have a low affinity of heparin/heparan sulfate and are easy to circulate in serum. Their biological function is exerted as both a growth factor binding FGFRs with co-receptors and as an endocrine molecule.

Many studies have demonstrated the significance of these FGFs and FGFRs in the development and progression of PCa. Herein, we discuss the current knowledge regarding the role of FGFs and FGFRs—including paracrine FGFs, endocrine FGFs, and FGFRs—in the development and progression of PCa, focusing on the representative molecules in each subfamily.

Thus, the FGF can significantly influence other cells and this is especially the case in cancer cells. FGF are but one class of growth factors[19]. Importantly the FGF act as both paracrine and endocrine. They can act closely and also at a distance. As we shall also note, this is the case for a variety of the cells in the EMT.

As will be noted, there are 18 such growth factors all possessing the ability to activate cells in a variety of ways; paracrine and endocrine. Now as Wesche et al have noted, the structure and complexity of the FGF family is also significant:

The FGF family consists of 18 ligands that bind to four homologous high-affinity FGFRs (FGFR1–FGFR4). The FGFs are secreted polypeptidic growth factors that bind to receptors expressed at the cell surface of target cells.

Most FGFs have signal sequences for secretion, except FGF1 and FGF2 that utilize a non-classical secretion pathway circumventing the ER (endoplasmic reticulum). In addition to the 18 secreted ligands that bind to cell-surface receptors, four members of the FGF family, the FHFs (FGF homologous factors), are not secreted and act intracellularly. The FGFRs have an overall structure similar to most RTKs. They are single-pass transmembrane proteins that consist of an extracellular part that binds FGF ligands, a transmembrane domain and an intracellular tyrosine kinase domain that transmits the signal to the interior of the cell.

[19] https://www.researchgate.net/publication/329702571_Growth_Factors_Pathways_and_Cancers

The intracellular kinase domain is similar to the VEGFR and PDGFR kinases in that it has an insert, resulting in a split kinase domain. The extracellular part is composed of three Ig-like domains (I–III) with an acidic, serine-rich region between domains I and II (termed the acid box). The first Ig-like domain is, together with the acid box, thought to play a role in receptor autoinhibition.

Domains II and III constitute the FGF ligand-binding site. In FGFR1–3, alternative splicing in Ig-like domain III creates isoforms with different ligand-binding specificities (FGFR1 IIIb–FGFR3 IIIb and FGFR1 IIIc–FGFR3 IIIc). The FGFR IIIb isoforms are predominantly epithelial and the IIIc isoforms are predominantly mesenchymal, with their corresponding ligands only activating either the epithelial or mesenchymal isoforms, except FGF1 which binds all receptor isoforms. Thus, paracrine signalling is achieved by, for instance, epithelial cells producing ligands that only activate the corresponding mesenchymal FGFR IIIc isoforms, and vice versa.

FGFs also bind to low-affinity receptors present on most cells, the HSPGs (heparan sulfate proteoglycans). HSPGs consist of a proteoglycan core that binds two or three linear polysaccharides (heparan sulfate chains). The FGFs bind to the negatively charged polysaccharides through electrostatic interactions. HSPGs both protect the ligands from degradation and are also involved in the complex formation between the FGFs and the FGFRs. Binding of FGFs to the receptors forces the dimerization of a ternary complex consisting of FGF, FGFR and heparan sulfate

From Yun et al we have the following summary Table as modified:

Function	***Subfamily related to the function***	***Target cell***
Cell Proliferation	FGF1, FGF2 FGF4	Preadipocyte Endothelial cell, epithelial cell, fibroblast cell, neural stem cell Trophoblast stem cell
Cell Proliferation	FGF7, FGF10	Epithelial cell
Cell Proliferation	FGF18	Osteoblast, chondrocytes, osteoclast
Cell Migration	FGF2	Astrocyte, myogenic cell
Cell Migration	FGF4	Myogenic cell
Cell Migration	FGF7	Epithelial cell, keratinocyte
Cell Migration	FGF8	Neural crest cell
Cell Differentiation	FGF1, FGF2	Neuroepithelial
Cell Differentiation	FGF7	Keratinocyte
Cell Differentiation	FGF20	Monkey stem cell
Angiogenesis	FGF1, FGF2	Endothelial cell

These are a brief summary of the FGR functions and targets. We shall examine these in the context of the fibroblast as well as the TME in toto.

4.3.6 FGFR

The FGF receptor, FGR plays a significant role in tumor development. The receptors are what takes the growth factor and then allows it to make the cell perform in a specific manner. As Acevedo et al have noted:

Fibroblast growth factor receptors (FGFRs) comprise a subfamily of receptor tyrosine kinases (RTKs) that are master regulators of a broad spectrum of cellular and developmental processes, including apoptosis, proliferation, migration and angiogenesis. Due to their broad impact, FGFRs and other RTKs are highly regulated and normally only basally active. Deregulation of FGFR signaling by activating mutations or ligand/receptor overexpression could allow these receptors to become constitutively active, leading to cancer development, including both hematopoietic and solid tumors, such as breast, bladder and prostate carcinomas.

Aberrant expression of multiple FGF family members and their cognate receptors are found in multiple cancers, including PCa, providing a strong indication of their role in neoplastic progression. While FGFR2 signaling is key in regulating both prostate morphogenesis and homeostasis, FGFR1 signaling has been more tightly correlated with PCa progression, evidenced by elevated expression of FGFR1 and some of its ligands in both human PCa and mouse prostate tumor models, such as SV40 T/t antigen (T/tag)- based TRAMP.

There have been a number of studies to date of the expression of FGFR1 in human PCa.12,16-19 Both our studies12 and those of Hamaguchi et al.20 demonstrate that in benign prostate glands, staining is seen primarily in cells in a basal location within the gland (encompassing basal cells, transit amplifying cells and prostatic progenitor cells) although the exact cell type expressing FGFR1 is unclear. All studies to date using immunohistochemistry (IHC) have shown increased expression of FGFR1 in PCa. …Thus, it is unequivocal that FGFR1 is increased in PCa. The linkage of FGFR1 to outcome is less clear.

In the case of melanomas, for example, Metzner et al have noted:

Cutaneous melanoma is a tumor with rising incidence and a very poor prognosis at the disseminated stage. Melanomas are characterized by frequent mutations in BRAF and also by overexpression of fibroblast growth factor 2 (FGF2), offering opportunities for therapeutic intervention. We investigated inhibition of FGF signaling and its combination with dacarbazine or BRAF inhibitors as an antitumor strategy in melanoma.

The majority of melanoma cell lines displayed overexpression of FGF2 but also FGF5 and FGF18 together with different isoforms of FGF receptors (FGFRs) Blockade of FGF signals with dominant-negative receptor constructs (dnFGFR1, 3, or 4) or small-molecule inhibitors (SU5402 and PD166866) reduced melanoma cell proliferation, colony formation, as well as anchorage-independent growth, and increased apoptosis.

DnFGFR constructs also significantly inhibited tumor growth in vivo. ***Combination of FGF inhibitors with dacarbazine showed additive or antagonistic effects, whereas synergistic drug interaction was observed when combining FGFR inhibition with the multikinase/BRAF inhibitor sorafenib or the V600E mutant-specific BRAF inhibitor RG7204.*** *In conclusion, FGFR inhibition has antitumor effects against melanoma cells in vitro and in vivo. Combination with BRAF inhibition offers a potential for synergistic antimelanoma effects and represents a promising therapeutic strategy against advanced melanoma. ….*

The use of BRAF inhibitors has been shown to have significant impact on melanomas, but not universally. This discussion points again towards the influence of the TME and particularly the FGF. They continue:

Overexpression of growth and survival-promoting factors is an important hallmark of neoplastic cells and a major driving force for tumor progression and dissemination. Expression of fibroblast growth factor 2 (FGF2) has been identified as an important characteristic of melanoma cells in contrast to normal melanocytes and has been linked to tumor progression in melanoma and multiple other malignancies.

The role of other FGFs is widely unexplored in melanoma so far. FGFs constitute a structurally conserved family of polypeptide growth factors, with 22 members in humans. FGFs transduce signals through binding to transmembrane receptor tyrosine kinases, named FGF receptors (FGFR1–4), and also bind with lower affinity to heparin-like glycosaminoglycans of the extracellular matrix. After ligand binding, FGFRs activate major cellular growth and survival pathways including, for example, mitogen-activated protein kinase and phosphoinositide 3-kinase signal cascades.

In addition, in a review paper by Wesche et al the authors summarize a multiplicity of cancers related to the FGFR. Specifically:

i. Breast
ii. Bladder
iii. Prostate
iv. Endometrial
v. Lung
vi. Myeloma
vii. Rhabdomyosarcoma
viii. EMS/SCLL (Leukemia)

Other putative cancers are also discussed.

4.4 NEUTROPHILS

Wu et al note:

The mobilization of neutrophils from bone marrow to tumor sites occurs in three phases including expansion and maturation of pre-mature neutrophils in the bone marrow,

intravasation to circulation through attachment to endothelial cells, and the chemotactic movement of neutrophils to tumor sites. The pre-mature neutrophils are derived from hematopoietic stem cells.

The proliferation and maturation of neutrophils require the regulation of granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF). The neutrophil maturation also includes the nuclear morphology change—the original round-shape nucleus finalizes to a segmented shaped nucleus and surface antigen expression changes including CD 65 and CD16 .

The release of neutrophils in bone marrow mainly depends on the interplay between CXCR4 and CXCR2 and its ligands. These two receptors belong to the CXC chemokine receptor family as G-protein coupled receptors. CXCR4 and CXCR2 are expressed on the surface of the neutrophil and span seven times the neutrophil membrane. The role of CXCR4 is for neutrophil homing in the bone marrow. Higher levels of CXCR4 and its ligands (for instance, CXCL12) will restrain the neutrophils mobility. An initial step for neutrophil movement is the disruption of CXCR4 and its ligand expression by factors including G-CSF. ...

Tumor-associated neutrophils are generally considered a pro-tumor factor in multiple tumor types, including breast cancer.

Using over 5000 cases of 25 different cancer types, Gentles et al. indicated that higher polymorpho-nuclear cell (PMN, including neutrophils) infiltration would lead to the lowest overall survival for those cancer patients compared to other leukocytes .

Additionally, the higher neutrophil to lymphocyte ratio (NLR) indicates a worse prognosis for those patients. There are also studies regarding neutrophils establishing a pre-metastatic niche for the malignant tumor cells. These studies indicate the overall pro-tumor functions of neutrophils in multiple cancer types.

Our focus herein is on neutrophils and thus this will be our initial discussion. All too often we consider them to be associated with infections. Yet they respond to a multiplicity of cellular disfunctions especially inflammation. Furthermore, as we shall discuss later they play a role in multiple cancers as a tumor associated neutrophil, TAN.

As Abbas et al note :

Phagocytes, including neutrophils and macrophages, are cells whose primary function is to ingest and destroy microbes and remove damaged tissues. The functional responses of phagocytes in host defense consist of sequential steps: recruitment of the cells to the sites of infection, recognition of and activation by microbes, ingestion of the microbes by the process of phagocytosis, and destruction of ingested microbes. In addition, through direct contact and by secreting cytokines, phagocytes communicate with other cells in ways that promote or regulate immune responses.

Blood neutrophils and monocytes, which differentiate into macrophages after entering tissues, are produced in the bone marrow, circulate in the blood, and are recruited to sites of inflammation. Although both are actively phagocytic, they differ in significant ways. The neutrophil response is more rapid and the lifespan of these cells after they enter tissues is short, whereas macrophages in tissues can live for long periods so that the macrophage response may last for a prolonged time.

Neutrophils mainly use cytoskeletal rearrangements and enzyme activation to mount rapid, transient responses, whereas macrophage responses rely more on induced gene transcription and protein expression. In addition, as we discuss later, there are subsets of macrophages that normally reside in healthy tissues, but neutrophils do not. The functions of phagocytes are important in innate immunity and also in the effector phase of some adaptive immune responses.

Neutrophils circulate as spherical cells approximately 12 to 15 μm in diameter with numerous membranous projections. The nucleus is segmented into three to five connected lobules. Because of their nuclear morphology, neutrophils are also called polymorphonuclear leukocytes (PMNs), to contrast them with mononuclear cells (macrophages and lymphocytes), whose nuclei are not multilobed. The cytoplasm contains two types of membrane-bound granules.

The majority of these granules, called specific granules, are filled with enzymes, such as lysozyme, collagenase, and elastase. These granules do not stain strongly with either basic or acidic dyes (hematoxylin and eosin, respectively), which distinguishes neutrophils from two other types of circulating leukocytes with cytoplasmic granules, called basophils and eosinophils. The remainder of the granules of neutrophils, called azurophilic granules because they are stained by azure A dyes, contain enzymes (e.g., myeloperoxidase) and microbicidal substances, including defensins and cathelicidins… Neutrophils are produced in the bone marrow and arise from precursors that also give rise to circulating monocytes.

Production of neutrophils is stimulated by granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). An adult human produces more than 1×10^{11} neutrophils per day, each of which circulates in the blood for a few hours or up to 5 days before dying. Neutrophils may migrate to sites of infection rapidly after the entry of microbes. After entering tissues, neutrophils function for only 1 to 2 days and most of them then die.

The above is a high-level discussion of neutrophils. A key observation is that neutrophils have a short lifetime and a significant generation rate.

We shall examine such questions as to where the CSFs come from and what activates the sources of proliferation and differentiation. We also will examine the details of the process of proliferation and differentiation.

4.4.1 Neutrophil Complexity

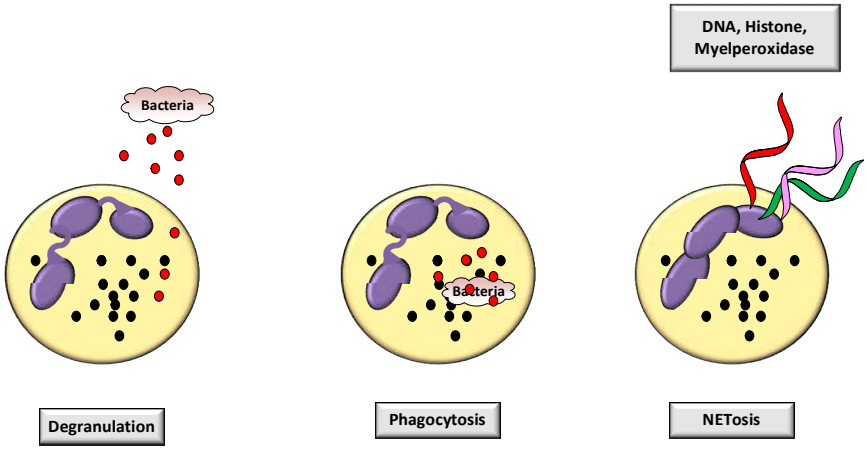
Neutrophils have a complexity comparable to many other HSC descendants. Understanding this complexity and what elements affect what cells in what environments is essential. Now Rosales has noted:

Neutrophils are the most abundant leukocytes in the circulation, and have been regarded as first line of defense in the innate arm of the immune system. They capture and destroy invading microorganisms, through phagocytosis and intracellular degradation, release of granules, and formation of neutrophil extracellular traps after detecting pathogens. Neutrophils also participate as mediators of inflammation. The classical view for these leukocytes is that neutrophils constitute a homogenous population of terminally differentiated cells with a unique function.

However, evidence accumulated in recent years, has revealed that neutrophils present a large phenotypic heterogeneity and functional versatility, which place neutrophils as important modulators of both inflammation and immune responses. Indeed, the roles played by neutrophils in homeostatic conditions as well as in pathological inflammation and immune processes are the focus of a renovated interest in neutrophil biology. In this review, I present the concept of neutrophil phenotypic and functional heterogeneity and describe several neutrophil subpopulations reported to date. I also discuss the role these subpopulations seem to play in homeostasis and disease

The above sets the stage for trying to understand the complexity of neutrophils. The classic view was one of a common neutrophil with a well understood path of proliferation and differentiation. We shall see that it is much more complex than that.

Rosales does also note in the case of bacterial attacks that neutrophils act in a variety of ways as shown below:



It is then noted that NETosis is defined as:

Three main antimicrobial functions are recognized for neutrophils: phagocytosis, degranulation, and the release of nuclear material in the form of neutrophil extracellular traps (NETs).

These functions were considered, until recently, the only purpose of neutrophils.

However, current research by investigators in several fields of neutrophil cell biology has revealed that neutrophils possess a much diverse repertoire of functional responses that go beyond the simple killing of microorganisms.

Neutrophils respond to multiple signals and respond by producing several cytokines and other inflammatory factors that influence and regulate inflammation and also the immune system.

Nowadays it is recognized that neutrophils are transcriptionally active complex cells that produce cytokines, modulate the activities of neighboring cells and contribute to the resolution of inflammation, regulate macrophages for long-term immune responses, actively participate in several diseases including cancer, and even have a role in innate immune memory. The multitude of neutrophil functional responses is induced by transcriptional activation and by changes in expression of surface molecules or activity. ...

NETosis, the process for producing NETs can be activated by multiple types of microorganisms.

Yet, the capacity of neutrophils to undergo NETosis can vary with physiological states, suggesting a neutrophil diversity that could be clinically relevant.

In fact, several reports indicate that NETs can influence thrombosis and vascular inflammation, cancer and autoimmunity.

As mentioned before some metabolic conditions associated with states of ***chronic inflammation****,* ***can increase neutrophil predisposition to form NETs****. Hence, neutrophils from diabetic patients and from systemic lupus erythematosus (SLE) patients have been shown to be more prone to NET formation.*

Our focus is chronic inflammation. Thus, the operation of NETosis is a critical one in understanding how neutrophils operate in such an environment such as osteoarthritis.

Likewise, Abbas et al note :

Neutrophils are produced in the bone marrow and arise from precursors that also give rise to circulating monocytes. Production of neutrophils is stimulated by granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). An adult human produces more than 1 × 1011 neutrophils per day, each of which circulates in the blood for a few hours or up to 5 days before dying. Neutrophils may migrate to sites of infection rapidly after the entry of microbes. After entering tissues, neutrophils function for only 1 to 2 days and most of them then die. ...

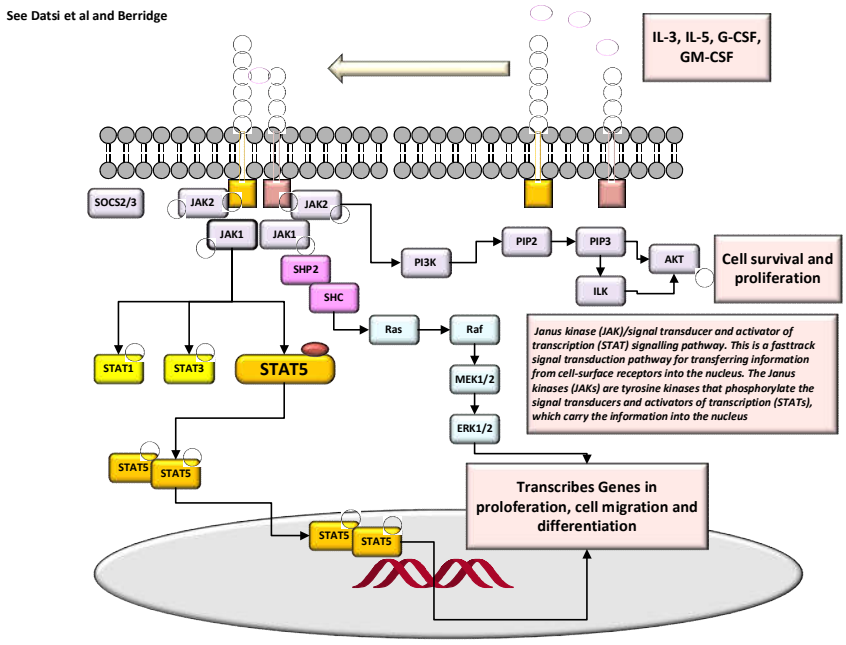
Macrophages promote the repair of damaged tissues by stimulating new blood vessel growth (angiogenesis) and synthesis of collagen-rich extracellular matrix (fibrosis). These functions are mediated by cytokines secreted by the macrophages that act on various tissue cells.

This then leads to the two key topics. Specifically, we now consider the control elements from a system perspective. The previous section examined the understanding of neutrophil proliferation and differentiation in situ, independent of the other forces effecting those changes. Here we consider the process in toto. First the process of proliferation and then differentiation.

As we shall note, proliferation is a key element. One needs many neutrophils in the event of an infection or inflammatory process. Yet the neutrophils must abate when the infection or inflammations dissipates. As we noted neutrophils have a short lifetime and thus their response to activate must be fast and likewise for deactivation. However, what we attempt to do here is to lay out the process of how an infection or inflammation drives proliferation and then how it is abated.

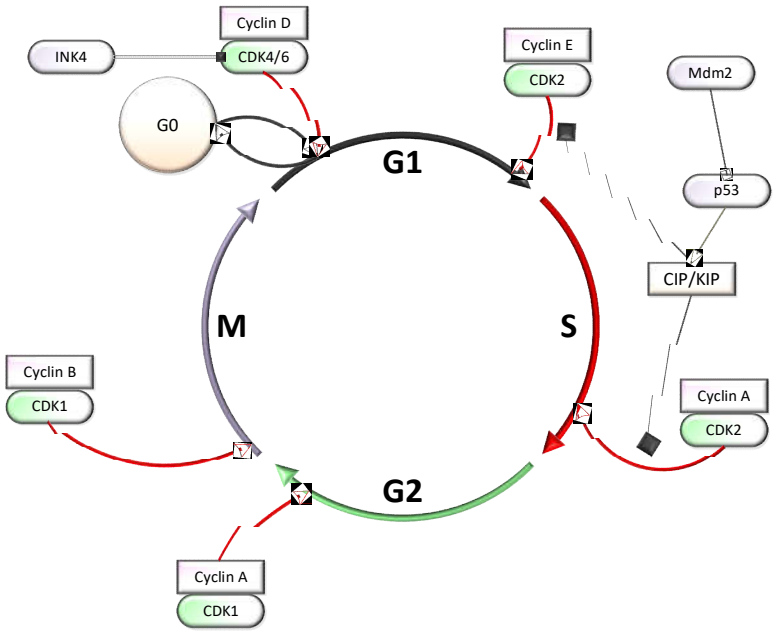
4.4.2 Proliferation

Proliferation is the expansion of the number of cells. In the following we show how G-CSF and other cytokines work through a cell to activate proliferation. The chart below is based on Datsi et al. The challenge is to understand why the progenitors of a mature neutrophil get activated. This chart focuses on the JAK/STAT paradigm, and we shall see variants of this.



How well this applies to the proliferation process details may still be an open issue. However, understanding this detail is essential for many therapeutic approaches.

Recall the details of the cell cycle are as below[20]:



Now c-Myc plays a key role here. Myc or specifically c-Myc, is a powerful gene element which induces cell growth. c-Myc is so strong promoter of cell proliferation and growth. c-Myc is a transcription factor which is essential in the growth and expansion of the cell.

As Pelengaris et al note regarding the impact of c-MYC:

The proto-oncogene c-MYC encodes a transcription factor that is implicated in various cellular processes, cell growth, proliferation, loss of differentiation and apoptosis.

c-MYC activates a variety of known target genes as part of a heterodimeric complex with the protein MAX.

For example, cyclin D2 and CDK2 are essential for cell-cycle progression, and translation initiation factors eIF4 and eIF2 are important in cell growth.

MYC/MAX heterodimers regulate gene activation through chromatin remodelling: association with co-activator TRRAP, which contains HAT activity, leads to acetylation of nucleosomal histones.

c-MYC inhibits the differentiation of many cell types. Conversely, MAD/MXI1 transcription factors promote differentiation by antagonizing c-MYC function by forming dimers with MAX. MAD?MAX dimers recruit corepressors (such as SIN3) and HDACs to target DNA, leading to histone deacetylation and subsequent repression of MYC target genes. c-MYC sensitizes cells to

[20] See Morgan for an excellent overview of all cell cycle issues.

a wide range of pro-apoptotic stimuli in vitro via cytochrome c release from mitochondria and subsequent formation of the apoptosome with APAF1 and procaspase-9.

Our interest in c-MYC is in its role in neutrophil proliferation. It has been found to be an essential step in terms of its activation. We shall discuss this later in some further detail.

As Madden et al note further details on c-MYC and its activation:

Myc is a transcription factor that belongs to the basichelix-loop-helix-leucine zipper (bHLHZip) family present in the cell nucleus, where it acts to regulate cell growth, differentiation, metabolism and death, and is frequently dysregulated in many human cancers. It is the prototype member of the Myc family that also encompasses N-Myc and L-Myc proteins in mammalian cells, all of which are highly homologous but distributed differently. c-Myc is ubiquitous and highly abundant in proliferating cells, whereas N-Myc and LMyc display more restricted expression at distinct stages of cell and tissue development. Myc proteins exist within the Myc/Max/Mxd network.

To fold and become transcriptionally active cMyc must first heterodimerize with Max, a process governed by the coiling of their bHLHZip domains. Once dimerized, the c-Myc/Max complex acts as a master transcriptional regulator by binding via its basic region to a specific DNA consensus sequence CANNTG, known as the Enhancer-box (E-box). Within the network, c-Myc can only heterodimerize with Max, whereas Max is more promiscuous and able to homodimerize or heterodimerize with other factors that share a bHLHZip motif. These include proteins of the Mxd family (Mxd1-Mxd4, formally called Mad proteins) as well as Mnt (a protein distantly related to Mxd-family), and the much larger Mga, an unusual protein that contains both a bHLHZip motif and a T-domain DNA-binding motif. …

c-Myc is a master regulator of immunometabolism and its dysregulation is implicated in inflammatory, autoimmune, metabolic and other non-cancerous disorders, although it remains poorly understood. The lack of an effective inhibitor that directly targets cMyc compromises studies investigating the potential of c-Myc inhibition as a therapeutic strategy to treat chronic diseases. Nevertheless, recent reports using indirect inhibitors or transgenic mice have shown some potential.

It was recently verified that c-Myc expression is upregulated in group 2 innate lymphoid cells (ILC2s) in the blood of asthma patients. Using a mouse model of allergic inflammation, it was found that inhibition of c-Myc repressed ILC2 activity, causing reduction in airways inflammation and other pathogenic responses

In the paper by Iwata et al the authors examine its influence during the development of PIN in the prostate. They state:

Lo-MYC and Hi-MYC mice develop prostatic intraepithelial neoplasia (PIN) and prostatic adenocarcinoma as a result of MYC overexpression in the mouse prostate. However, prior studies have not determined precisely when, and in which cell types, MYC is induced. Using immunohistochemistry (IHC) to localize MYC expression in Lo-MYC transgenic mice, we show

that morphological and molecular alterations characteristic of high-grade PIN arise in luminal epithelial cells as soon as MYC overexpression is detected.

These changes include increased nuclear and nucleolar size and large-scale chromatin remodeling. Mouse PIN cells retained a columnar architecture and abundant cytoplasm and appeared as either a single layer of neoplastic cells or as pseudo-stratified/multilayered structures with open glandular lumina—features highly analogous to human high-grade PIN.

Also using IHC, we show that the onset of MYC overexpression and PIN development coincided precisely with decreased expression of the homeodomain transcription factor and tumor suppressor, Nkx3.1. Virtually all normal appearing prostate luminal cells expressed high levels of Nkx3.1, but all cells expressing MYC in PIN lesions showed marked reductions in Nkx3.1, implicating MYC as a key factor that represses Nkx3.1 in PIN lesions.

To determine the effects of less pronounced overexpression of MYC we generated a new line of mice expressing MYC in the prostate under the transcriptional control of the mouse Nkx3.1 control region. These ''Super-Lo-MYC'' mice also developed PIN, albeit a less aggressive form. We also identified a histologically defined intermediate step in the progression of mouse PIN into invasive adenocarcinoma. These lesions are characterized by a loss of cell polarity, multi-layering, and cribriform formation, and by a ''paradoxical'' increase in Nkx3.1 protein. Similar histopathological changes occurred in Hi-MYC mice, albeit with accelerated kinetics.

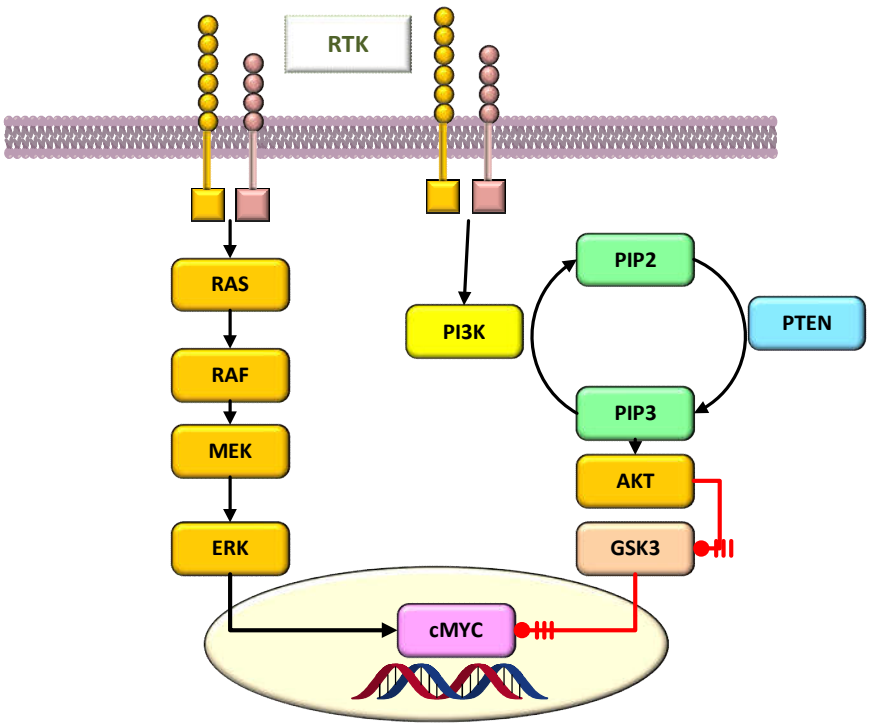
Our results using IHC provide novel insights that support the contention that MYC overexpression is sufficient to transform prostate luminal epithelial cells into PIN cells in vivo. We also identified a novel histopathologically identifiable intermediate step prior to invasion that should facilitate studies of molecular pathway alterations occurring during early progression of prostatic adenocarcinomas.

In the following graphic we depict the influence elements on c-Myc. This is a complex system of interlinking genes which when expressed in the correct manner can slow cell over expansion. The chart below is a modification from Bunz (p. 203) and it shows the gross characteristics of this control path. PTEN is a key element in control. What this does not show are two key elements, and indirectly a third.

First it does not show the fact that these are protein concentrations at work, one influencing the other and so forth. There is a feedback mechanism missing.

Second, it does not portray the temporal elements, namely this is a static gross representation of the influencing factors as if done in some generic snapshot. I fact the concentrations are time varying and it is this time variation which when combined with the feedback loops renders certain instabilities leading to malignancy, namely uncontrolled growth.

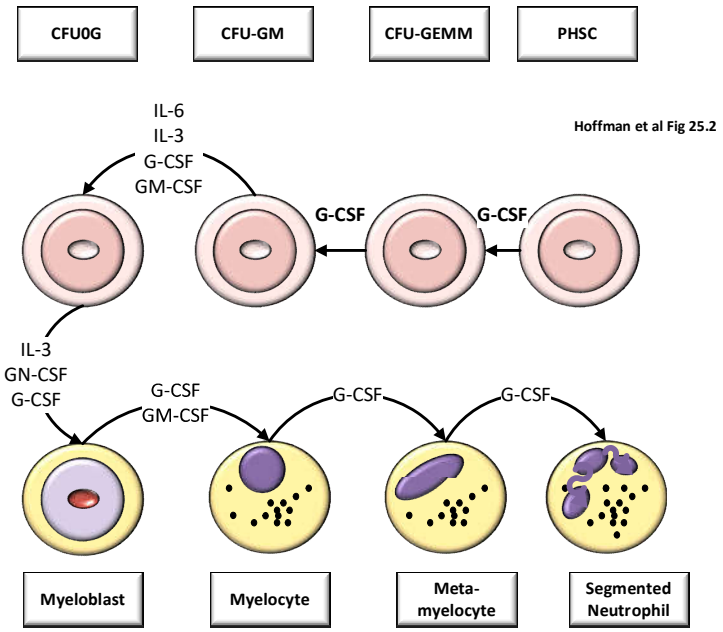
Third, it fails to show the other genes and specifically the feedback mechanism of these genes. Namely PTEN is influence by these.



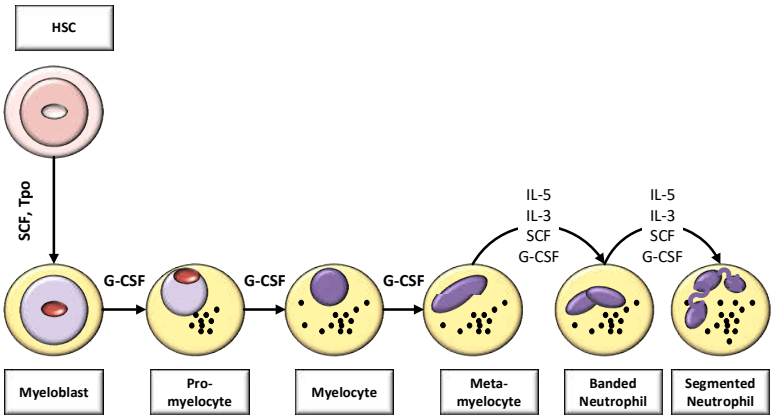
Thus, cMYC plays a key role but it is a role that is played in may environments. Specifically, the cell cycle and the drivers of that cycle can be initiated by cMYC actions.

4.4.3 Differentiation

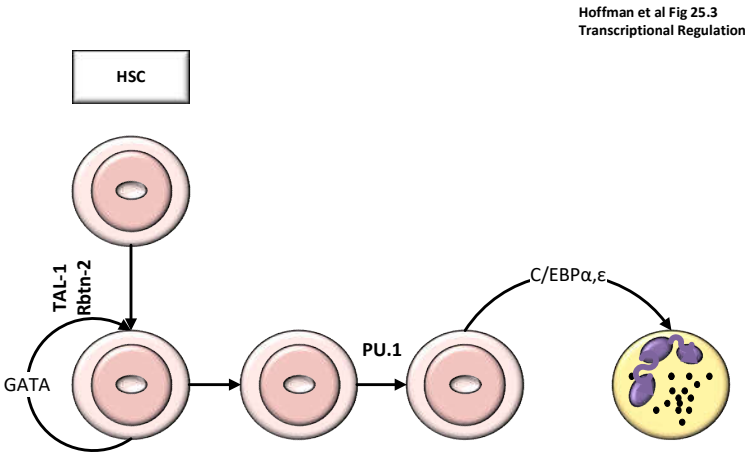
Now the details of differentiation seem better understood. One starts with the HSC and then goes through certain expression and morphological changes. The flow described below provides some insight. This is a modified version as presented by Hoffman. It should be noted that there are a multiplicity of steps in differentiation leading to the release of multi-lobed neutrophils.



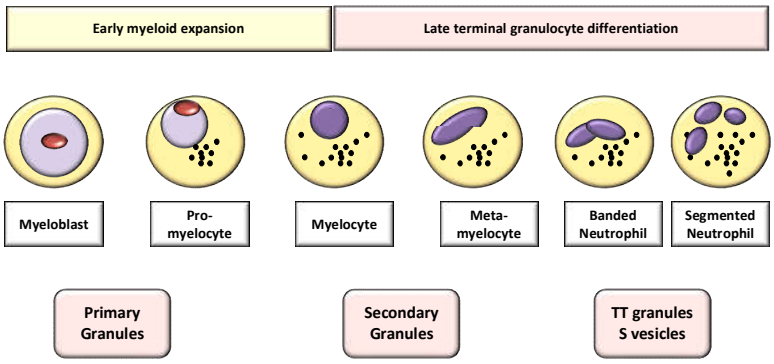
The diagram below presents another view of the process but focusing on the more mature version. Again G-CSF plays the controlling element. We will focus on G-CSF later in this section.



Further delineation is presented in the following figure where we see the development of the granulation. As these processes move forward a variety of CD surface markers can be used to ascertain the progress.

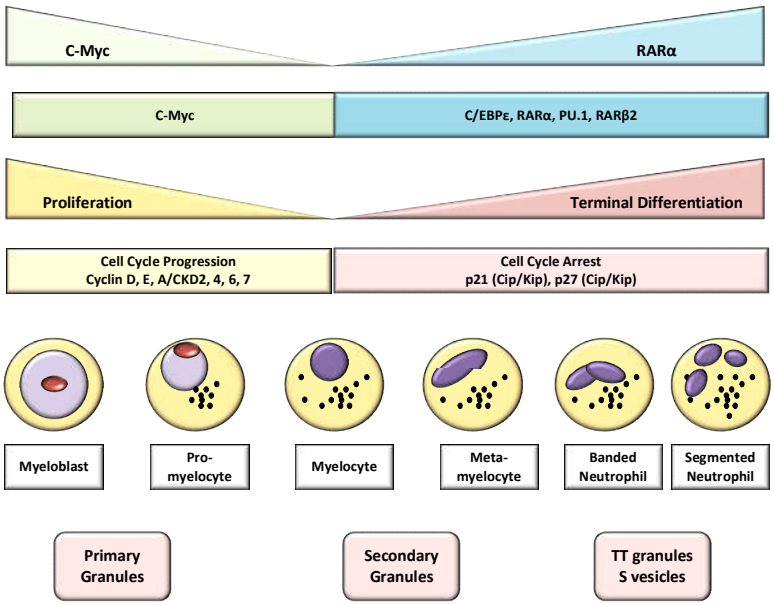


Another view is from Abdel-Azim et al. Here the authors delineate the proliferation and differentiation steps in some detail.



Now the following details the change from Proliferation to Differentiation. It is the activation of c-Myc that plays a key role in proliferation. Significant cell cycle activation allows for the proliferative process whereas that is tampered down with differentiation taking over on the proliferated cells.

From Abdel-Azim et al we have the following progression detailed.



These steps represent a complex set of interactions that are still a work in progress. These steps also show that there are multiple variants understood along the way. It further argues that proliferation seems to be a distributed process. If so then one may ask how if there is an excess drive on neutrophils why there will not be a commensurate proliferation of other common pathway granulocytes.

There is a question of uniqueness in proliferation that seems to have been unanswered. If G-CSF drives neutrophils from the Myeloblast stage forward, one should expect that all resulting myeloid lines should proliferate. If we have neutrophilia alone then there must be some complex set of factors that differentiate at the same time as proliferation. Thus, if that is the case then the above graphic displays a dichotomy which does not exist. What then is the actual process?

As von Vietinghoff and Ley have summarized some minor details:

Hematopoietic cytokines promote neutrophil progenitor proliferation and differentiation acting in a complex network. The major cytokine for neutrophil proliferation and survival is GCSF. Mice and humans deficient in either G-CSF or its receptor suffer from profound neutropenia. G-CSF currently is the major therapeutic agent for neutropenia of iatrogenic as well as genetic and various other origins.

Extensive basic science and clinical data exist on the role of other granulopoietic cytokines such as M-CSF, GM-CSF, interleukin (IL)-6, IL-3, IL-17 and, most recently, IL-22 that have been reviewed elsewhere in detail. Genetic modification of intracellular messengers downstream of GCSF in mice elucidated their stage-specific roles.

For example, both STAT3 and SOCS3 deficiency resulted in neutrophilia and an increased pool of late-stage progenitors in the bone marrow thus implicating an inhibitory role.

The role of transcription factors and microRNA in neutrophilic differentiation has recently been reviewed. A number of monogenic defects associated with rare forms of congenital neutropenia in humans are known.

Maturation arrest and increased cell death of neutrophil progenitor proliferation have been observed in humans with elastase gene mutations, but also in genes encoding a number of transcription factors such ***as Growth factor independent 1 (GFI 1[21]), HCLS1[22] associatied protein X-1 (HAX1[23]), and lymphoid enhancer factor-1 (LEF-1[24])…***

The above is a complex set of adjuvant genes and their protein elements. The detailed interaction amongst these products is specified but not well defined. They continue:

Stable neutrophil blood counts are the result of a highly dynamic feedback system. The study of genetically altered mice and monogenic diseases in humans has given insight into some of the involved mechanisms. However, neutrophil counts in healthy humans are regulated by a variety of environmental and genetic factors, most of which remain currently unknown. As elevated counts within the normal range are associated with excess mortality, elucidation of factors involved in steady state neutrophil regulation might have clinical relevance.

4.5 NK CELLS

[21] As NCBI notes: *This gene encodes a nuclear zinc finger protein that functions as a transcriptional repressor. This protein plays a role in diverse developmental contexts, including hematopoiesis and oncogenesis. It functions as part of a complex along with other cofactors to control histone modifications that lead to silencing of the target gene promoters. Mutations in this gene cause autosomal dominant severe congenital neutropenia, and also dominant nonimmune chronic idiopathic neutropenia of adults, which are heterogeneous hematopoietic disorders that cause predispositions to leukemias and infections. Multiple alternatively spliced variants, encoding the same protein, have been identified for this gene.*

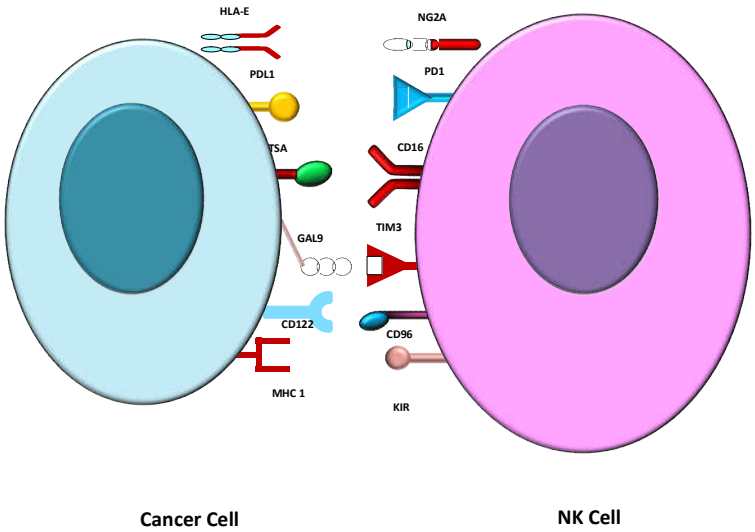
[22] As NCBI notes: *Enables RNA polymerase II-specific DNA-binding transcription factor binding activity and protein kinase binding activity. Involved in several processes, including positive regulation of intracellular signal transduction; positive regulation of protein phosphorylation; and regulation of transcription, DNA-templated. Located in cytosol; nucleus; and plasma membrane. Part of transcription regulator complex*.

[23] As NCBI notes: *The protein encoded by this gene is known to associate with hematopoietic cell-specific Lyn substrate 1, a substrate of Src family tyrosine kinases. It also interacts with the product of the polycystic kidney disease 2 gene, mutations in which are associated with autosomal-dominant polycystic kidney disease, and with the F-actin-binding protein, cortactin. It was earlier thought that this gene product is mainly localized in the mitochondria, however, recent studies indicate it to be localized in the cell body. Mutations in this gene result in autosomal recessive severe congenital neutropenia, also known as Kostmann disease. Two transcript variants encoding different isoforms have been found for this gene*.

[24] As NCBI notes: *This gene encodes a transcription factor belonging to a family of proteins that share homology with the high mobility group protein-1. The protein encoded by this gene can bind to a functionally important site in the T-cell receptor-alpha enhancer, thereby conferring maximal enhancer activity. This transcription factor is involved in the Wnt signaling pathway, and it may function in hair cell differentiation and follicle morphogenesis. Mutations in this gene have been found in somatic sebaceous tumors. This gene has also been linked to other cancers, including androgen-independent prostate cancer. Alternative splicing results in multiple transcript variants*

Natural Killer cells are part of the innate system. They have the capacity to attack tumors. The graphic below depicts the ligands and receptors between a cancer cell and NK cells. Often NK cells may react with early cancers and eliminate them. However, this capability may not endure.

See Carotta



As Yu notes:

Neoantigen peptides are presented to immune cells through the MHC class I (MHC-I) molecules on the surface of the cancer cell, distinguishing them from their normal counterparts (nonself). This presentation leads to the activation of T cells to become specific cytotoxicity CD8+ T cells, which can kill those tumor cells. NK cells can detect and rapidly destroy tumor cells with downregulated MHC-I expression, terming 'missing self' recognition.

Hereafter, NK cells quickly produce cytokines and secret the interferon-γ (IFNγ), tumor necrosis factor-alpha (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF), and chemokines for activating other immune cells such as T and B cells to boost adaptive immunity. Moreover, NK cells can be provoked by antibody opsonization for mediating antibody-dependent cellular cytotoxicity (ADCC).

To trigger and develop the entire immune response requires the interaction of tumor cells and immune cells, which needs three signals: first signal—an antigen expressed in cancer cells; second signal—stimulatory molecules in both cancer and immune cells, and third signal—cytokine signaling in immune cells. However, cancers can develop multiple strategies to escape immune cell attack.

Immune escape is usually associated with loss of stimulating molecules that includes the downregulation of classical HLA molecules (missing self-hypothesis), loss of stimulatory cytokines, and/or gain of suppressing molecules such as expression of nonclassical HLA-G,

functional Th2-type activity shift (e.g., decrease in IFNγ and/or increase in TGFb, IL6, and IL10) and elevation of the Fas ligand on cancer cells. The interactivity of the immune cells and cancer cells can determine the fate of tumors. This can be described as three "E's", which are eradication, equilibrium, and escape. This process, called cancer immunoediting, has built a theoretical hypothesis for comprehending our immune protection and sculpting cancer immunogenicity on tumor progression. In early cancer development, tumor cells can be eliminated by the immune system via the responses of innate and adaptive immunity in the eradication phase.

However, tumor cells can manage the immune pressure to survive and then may enter the equilibrium phase. Escape is the phase of the tumor progression, where tumors are sculpted immunologically with the ability to inhibit the immune cells attach and establish an immunosuppressive tumor microenvironment (TME), then become clinically relevant as a mass and/or metastasis. Boosting immunity and inhibiting immunosuppressive TME are principal strategies for anticancer immune therapy.

One of the breakthroughs in antitumor immunotherapy focuses on utilizing adaptive immunity, achieving primary success through inhibiting immune checkpoints or using CAR-T cells to enhance antitumor CD8+ T cell responses. Emerging evidence indicates that tumors can develop various strategies to evade CD8+ T cell recognition, and side effects remain in T cell therapies. Although T cells can eliminate most cells within a primary tumor, a fraction can still escape antitumor immunity and survive, ultimately leading to metastatic disease and death of the patient. However, NK cells can preferentially attack these tumors. NK cells have built-in safety characteristics to avoid autoimmunity and have the ability to maintain immune homeostasis to prevent autoimmune disease. Moreover, a subgroup of NK cells can present memory-like recall responses, playing the role of adaptive immunity.

Thus, these unique features of NK cells make them attractive targets for immunotherapy that can provoke their potent antitumor mechanisms *(Table 1). Consequently, a new field of NK cells in metastasis has highlighted the important role of NK cells in immunosurveillance against metastasis. This review focuses on the critical functions of NK cells in metastasis and the recent development of novel and objectively effective cancer immunotherapies.*

4.6 TME Remediation

Bejarano et al discuss the strategies to remediate the TME blockage. They conclude:

Therapeutic targeting of the TME has long been viewed as a promising strategy in the anticancer armamentarium. The clinical approval of drugs and cell-based therapies directed toward the blood vasculature, immune checkpoints, and T cells has driven the continued exploration of the TME for additional targets to exploit. In this review, we have highlighted some examples of the successes of TME therapies, as well as those that have not lived up to initial expectations.

Even the success stories, such as ICIs, are still only beneficial for a subset of cancers and a minority of patients, and a major mechanism limiting their efficacy is an immunosuppressive

TME. Thus, the TME field will inevitably continue to focus its efforts on developing strategies to relieve immunesuppressive mechanisms, to activate antitumor immunity and/or boost the efficacy of immune-targeted agents. Given that immune suppression is mediated via diverse mechanisms and often interconnected cell types, strategies to identify and selectively target key vulnerabilities will be critical. Several areas hold considerable promise, including the modulation of the tumor vasculature in combination with immunotherapies.

One recent example involved inhibition of the PAK4 enzyme (which is selectively expressed in glioblastoma blood vessels) in combination with delivery of CAR T cells engineered to target the EGFRvIII mutation in glioma cells. In preclinical models, this therapy resulted in reprogramming of the vasculature, which promoted immune cell adhesion and engineered T cells' subsequent ability to enter the brain, thereby eliciting a robust antitumor response.

Another intriguing study in mouse colorectal cancer models showed that adaptive resistance to chemotherapy (5-FU and cisplatin) is associated with a pronounced stromal response and T-cell exclusion. Combined targeting of the desmoplastic stroma along with the vasculature (VEGFANG2) and a CD40 agonist resulted in a conversion from fibrotic immune-excluded tumors to enable the unleashing of a CTL-mediated anticancer response. These brief illustrative examples demonstrate the potential for such complex multitargeting strategies to be evaluated in animal models as a means to identify and stratify combinations for potential translation to patients.

This type of preclinical "prescreening" will be critical given the immense number of planned immunotherapy clinical trials in which there are simply not enough patients to enroll for all the foreseen combinations. In this regard, rational stratification of patients in advance and accurate monitoring of the immune response and other parameters while on trial will be invaluable to gain as much dynamic information as possible for responders versus nonresponders. Several recent advances highlight the power of such approaches. As an example, noninvasive liquid biopsies are routinely used to measure circulating tumor DNA in the blood; this was recently combined with peripheral immune analyses to predict clinical benefit from ICIs in patients with NSCLC while on treatment. Another tractable approach is the use of EVs as diagnostic markers, given their high accessibility (they are present in nearly all body fluids), and the fact that their molecular content highly depends on the cell of origin, thereby providing relevant information about the pathologic state of the EV-producing cells. Given that both the level of EVs/ exosomes and their content can be correlated with multiple clinical, several trials are evaluating EVs as biomarkers.

Other studies have incorporated the collection of patient tissue biopsies, such as in breast cancer, where the tumor immune microenvironment was assessed in samples collected before treatment, after the first cycle of neoadjuvant chemotherapy, and at the time of surgery. Interestingly, this longitudinal study revealed that the on-treatment immune response was more predictive of treatment outcome compared with the paired baseline samples, supporting the inclusion of these types of dynamic real-time analyses in clinical trials wherever possible. Looking forward, there are several areas of active investigation that will likely reveal further exciting insights into the TME in the coming years.

It will be critical to move beyond the current focus on targeting individual cell types of interest and rather adopt a more comprehensive systems-level approach in which we analyze and integrate all TME components to identify and disable the critical nodes. We now recognize that the TME can differ quite profoundly from one organ to another, and thus we cannot simply extrapolate findings between different tumor types. We must additionally examine the patient as a whole, and not only focus on the tumor in isolation. For instance, it will be essential to investigate how systemic influences, for example the gastrointestinal microbiome, metabolism, diet, or exercise, or underlying conditions, for example inflammation, cachexia, obesity, and aging, can alter the TME and affect treatment response. By leveraging this ever-expanding wealth of information, the long-held potential of targeting the TME for the benefit of a much broader population of patients diagnosed with cancer is now a goal that is finally within our reach.

5 VACCINE OPTIONS

We now consider vaccine options. Namely what are we sending into the system to facilitate the production of an immune response?

5.1 THE PARADIGM

We first examine the purpose, in our words the paradigm, assumed by a vaccine. Namely what is it supposed to do? As Gupta et al note regarding the current cancer vaccine paradigms:

For a therapeutic cancer vaccine, it is a prerequisite that it triggers a strong immunological reaction, precisely recognizes, and gets rid of tumor cells (primary and secondary), is antigen-specific, has minimal systemic side effects, and does not generate autoimmune responses.

Another consideration is that the vaccine must induce a robust immunological recall to counteract cancer cells, which is critical to attain long-term disease resolution. ***In reality, relapses, rather than the primary tumor, have been largely blamed for the high cancer mortality rate.***

The aim of immunotherapy-based cancer vaccines is to activate the endogenous cellular- or humoral-acquired immune system against cancer.

Mostly, cancer vaccines induce the production of cancer-specific CD8+ T-cells that specifically recognize and kill cancer cells.

Tumor antigen-specific cytotoxic T lymphocytes (CTLs) recognize cancer antigen epitopes by binding to their T-cell receptor (TCR).

Furthermore, CTLs via several TCR signaling pathways, such as degranulation (release of perforin/serine protease), or via upregulation of cluster of differentiation ligand (CD95L) or TNF-related apoptosis-inducing ligand (TRAIL), induce cancer cell death.

For effective use, CTLs need to be trained by tumor dendritic cells (DCs).

Type 1 conventional CD103+ migrating DCs are antigen-presenting cells (APCs) that elucidate CTLs before cancer cell detection via three different mechanisms: cancer antigen adhered to MHC-I, co-stimulatory molecules (CD80/86 and CD28/152), and pro-inflammatory cytokines (IL-12 and TNF-α).

CTLs and CD4C Th cells develop certain characteristics upon activation that greatly influence the subsequent efficiency of CTL cytotoxic responses.

In addition, cytokine-mediated DC licensing activates and supports CD4+ Th cells. APCs also activate CD4+ T-cells similarly to CD8+ T-cells, except that the tumor antigen epitope is displayed on MHC-II rather than MHC-I. CTLs and CD4C Th cells develop certain

characteristics after activation, that greatly influence the subsequent efficiency of CTL cytotoxic responses.

CTL phenotypes are commonly defined by the cytokine cocktail that is released via a cytotoxic mechanism to promote cell death. Many studies have demonstrated that CTL-mediated production of IFN Vaccines … production of IFN-Ɣ and TNF-α corresponds to good tumor reduction potential and improved patient endurance. Other investigations have shown that when CD4C Th cells adopt a Th1 phenotype, characterized by the release of IFN-Ɣ, TNF-α, and IL-2, patient endurance improves.

Although more debatable, it has been demonstrated that combining the Th1 response with a Th17 inclination, as defined by IL-17 production, may be even more advantageous. As each T-cell has a unique TCR that recognizes just one antigenic epitope, immunological responses that create a broad measure of antitumor T-cell levels (many T-cell clones) are stronger. The optimum immune response to immunization may vary amongst malignancies.

Cancer vaccination can also harness antibody-mediated cytotoxic pathways to limit cancer progression (Figure 3). Antibody-mediated cytotoxicity and antibody-mediated phagocytosis can be used to kill cancer cells when they bind to antibodies. Cancer vaccines based on humoral immunotherapy, aiming to elicit anticancer antibodies in the patient´s body, have mainly used these techniques for passive immunotherapy.

Immunological cells responsible for innate immunity (natural killer cells, macrophages, and neutrophils) can identify the attached antibody Fc receptors and drive cell lysis or phagocytosis, once antibodies recognize epitopes on cancer cell surfaces. and TNF-α corresponds to good tumor reduction potential and improved patient endurance.

Other investigations have shown that when CD4C Th cells adopt a Th1 phenotype, characterized by the release of production of IFN-Ɣ and TNF-α and IL-2, improved patient endurance. Although more debatable, it has been shown that combining the Th1 response with a Th17 inclination, as def even more advantageous. As each T-cell has a unique T genic epitope, immunological responses that create a broad measure of antitumor T-cell levels (many T-cell clones) are stronger. The optimum immune response to immunization may vary amongst malignancies.

Cancer vaccination can also harness antibody mediated cytotoxic pathways to limit cancer progression. Antibody mediated cytotoxicity and antibody-mediated phagocytosis can be used to kill cancer cells when they bind to antibodies.

Cancer vaccines based on hum elicit anticancer antibodies in the patient´s body, have passive immunotherapy. Immunological cells r (natural killer cells, macrophages, and neutrophils) can receptors and drive cell lysis or phagocytosis, once anti car cell surfaces., TNF-α, and IL-2, patient endurance improves. Although more debatable, it has been demonstrated that combining the Th1 response with a Th17 inclination, as defined by IL-17 production, may be even more advantageous.

As each T-cell has a unique TCR that recognizes just one antigenic epitope, immunological responses that create a broad measure of antitumor T-cell levels (many T-cell clones) are stronger. The optimum immune response to immunization may vary amongst malignancies. Cancer vaccination can also harness antibody-mediated cytotoxic pathways to limit cancer progression. Antibody-mediated cytotoxicity and antibody-mediated phagocytosis can be used to kill cancer cells when they bind to antibodies. Cancer vaccines based on humoral immunotherapy, aiming to elicit anticancer antibodies in the patient´s body, have mainly used these techniques for passive immunotherapy.

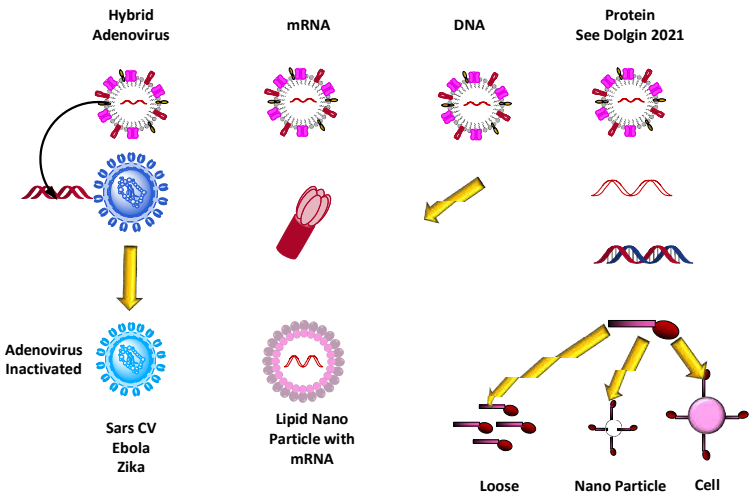
Immunological cells responsible for innate immunity (natural killer cells, macrophages, and neutrophils) can identify the attached antibody Fc receptors and drive cell lysis or phagocytosis, once antibodies recognize epitopes on cancer cell surfaces.

Finally, coactivation of other innate immunity systems, such as T-cells, can help to enhance the adaptive immune response that is sought by cancer vaccinations.

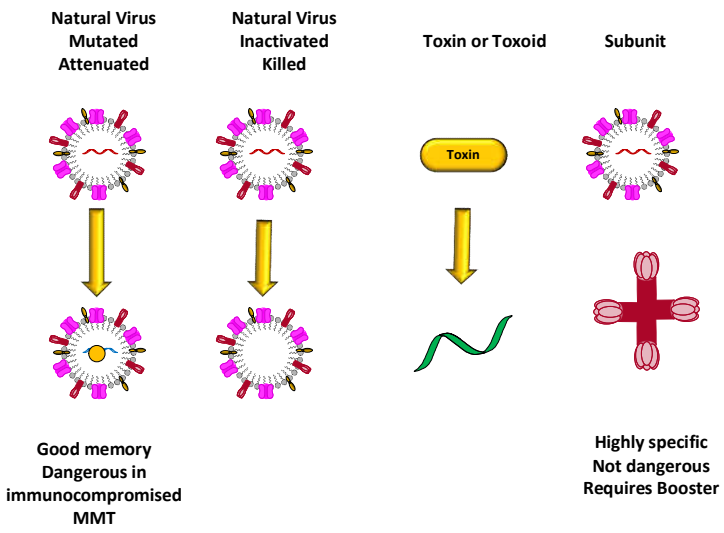
The innate lymphoid cells (ILCs), for example, NK cells or ***invariant NK T-cells (iNKT),*** *offer complementary abilities to CTLs in terms of cancer cell control. To avoid T-cell identification, cancer cells that downregulate MHC-I or overstimulate NK cell-activating receptors (e.g., NKG2D, 4-1BB) can be lysed by NK cells, which have cytotoxic capabilities.*

When iNKT cells are activated, they secrete cytokines such as Th1 or Th2 in the surroundings and enhance the expression of CD40L. The importance of iNKT cells in influencing adaptive immune responses has been demonstrated by their ability to aggressively boost DC and B-cell maturation, as well as indirectly promoting T-cell responses.

Despite this, cancer vaccinations generally fail to target NK or iNKT cells because they do not bear epitopes.



A second view is presented below:



We now proceed to consider the various vaccine options.

5.2 Cell

Cell based vaccines are probably the oldest form. Typically, the infecting virus is made non infective, say by using formaldehyde, then injected into the patient. Thus, the patient receives a compels set of Ag which are specific. Many classic vaccines use this approach. It does not require any specific knowledge of the Ags but it does require growth of the pathogen and effective neutralization.

5.3 DNA

DNA vaccines require some form of reverse transcriptase. They are more stable than mRNA but are subject to DNA changes and may be dangerous.

5.4 mRNA

As a result of the recent Corona virus pandemic the use of mRNA vaccines has expended greatly. RNA is more stable than DNA and in addition its proliferation in the cell does not require entry into the nucleus and possible DNA disruption.

5.5 Peptide

Peptide vaccines use small peptide sequences unique to the pathogen and are then injected to create an immune response.

5.6 Virus

Vaccinia has recently been adjudged a reasonable vaccine and as noted by Guo et al,

*… recombinant **vaccinia viruses (VV)** hold great promise as interventional agents. In this article, we first summarize the current understanding of virus biology and viral genes involved in host-virus interactions to further improve the utility of these agents in therapeutic applications. We then discuss recent findings from basic and clinical studies using VV as cancer vaccines and oncolytic immunotherapies.*

Despite encouraging results gleaned from translational studies in animal models, clinical trials implementing VV vectors alone as cancer vaccines have yielded largely disappointing results.

However, the combination of VV vaccines with alternate forms of standard therapies has resulted in superior clinical efficacy.

For instance, combination regimens using TG4010 (MVA-MUC1-IL2) with first-line chemotherapy in advanced-stage nonsmall cell lung cancer or combining PANVAC with docetaxel in the setting of metastatic breast cancer have clearly provided enhanced clinical benefits to patients.

Another novel cancer vaccine approach is to stimulate anti-tumor immunity via STING activation in Batf3-dependent dendritic cells (DC) through the use of replication-attenuated VV vectors.

Oncolytic VVs have now been engineered for improved safety and superior therapeutic efficacy by arming them with immune-stimulatory genes or pro-apoptotic molecules to facilitate tumor immunogenic cell death, leading to enhanced DC-mediated cross-priming of T cells recognizing tumor antigens, including neoantigens. Encouraging translational and early phase clinical results with Pexa-Vec have matured into an ongoing global phase III trial for patients with hepatocellular carcinoma. Combinatorial approaches, most notably those using immune checkpoint blockade, have produced exciting pre-clinical results and warrant the development of innovative clinical studies.

Finally, we discuss major hurdles that remain in the field and offer some perspectives regarding the development of next generation VV vectors for use as cancer therapeutics.

6 ADJUVANTS

Adjuvants are additives which enhance the immunity of the core vaccine elements. A classic one is aluminum salts which has been employed for a century.

6.1 TYPES

As Facciolo et al note:

Adjuvants are defined as constituents added to vaccines in order to improve immune responses towards an antigen. In addition, adjuvants have several benefits, such as the reduction in the antigen amount per vaccine dose and the number of vaccination sessions, and in certain cases, they increase the stability of the antigen component, extending its half-life and indirectly improving its immunogenic power Adjuvants can be grouped according to different criteria, such as their physicochemical properties, origins, and mechanisms of action. One of the most followed classification systems is the one based on their mechanisms of action, dividing them into two main categories: delivery systems (particulate) and immune potentiators.

A further class of adjuvants is mucosal adjuvants, a group of compounds that shares some features with the previous ones. In delivery system adjuvants, antigens are associated with an adjuvant that works especially as an antigen carrier. In addition, they are able to induce a local proinflammatory response by activating the innate immune system, leading to the recruitment of immune cells to the site of injection. Specifically, the antigen-adjuvant complex activates pattern-recognition receptor (PRR) pathways by acting as pathogen-associated molecular patterns (PAMPs). This causes the activation of innate immune cells with the production of cytokines and chemokines. The same pathway is directly activated by immunopotentiators.

The addition of adjuvants is particularly useful for vaccines used in the elderly due to the physiological phenomenon of immunosenescence occurring in this category of subjects, which is responsible for the reduction of immune responses after natural infections or artificial stimuli (vaccinations). In this case, the presence of adjuvants can represent a valid tool to overcome this limit in the use of vaccines. Moreover, adjuvants are particularly useful for subunit vaccines that are often too weak to stimulate a robust immune response alone.

However, not all vaccines need adjuvants. For example, licensed conjugated meningococcal vaccines do not contain adjuvants because the conjugation itself with a protein carrier is able to stimulate a good immune response …

The adjuvant properties of aluminium salts were discovered in the 1920s, and these compounds have been used as vaccine adjuvants since 1926. The use of aluminium salts added to growth media was originally considered in order to induce the precipitation of tetanus and diphtheria antigens and therefore to help their purification. However, it was immediately evident that these aluminium-precipitated antigens showed more immunogenicity than the soluble ones. Therefore, aluminium salts are the adjuvants that have been used for the longest period of time and the most frequently included in vaccines, with about one-third of currently licensed vaccines containing

aluminium. Asa result, aluminium salts are the most tested in terms of safety among the vaccine adjuvants.

6.2 DELIVERY

As Facciolà et al note the delivery systems include:

1) Mineral salts
2) Emulsions
3) Micro Particles including virus like particles

As Paston et al noted:

6.2.1 Electroporation and Gene Gun Vaccine Delivery

There have been significant improvements in optimizing vaccine administration routes to overcome poor cellular uptake. Also, improvements with delivery and plasmid design have improved the efficacy of DNA vaccines in both pre-clinical and clinical studies. One strategy for improving the uptake of plasmid DNA into antigen presenting cells (APCs) is by using electroporation. Electroporation delivers small electrical pulses that causes transient pores to form in the cell membrane. During the period of membrane destabilization plasmid DNA present in the extracellular environment around the target cell gains access to the intracellular compartment

6.2.2 Nanoparticle Vaccine Delivery Systems

Nanoparticle based drug delivery platforms offer an alternative vehicle for delivering drugs that have previously suffered from pharmacokinetic limitations including poor bioavailability, a short half-life or poor solubility. A variety of nanoparticles have been explored as delivery systems or as , such as polymeric nanoparticles, liposomes, micelles, carbon nanotubes, mesoporous silica nanoparticles, gold nanoparticles and virus nanoparticles, that can all be used alone or in combination .Liposomes are a popular nanoparticle vaccine delivery system, they are versatile and can be constructed with a variety of different properties by changing the lipid composition, charge, size and surface properties

6.2.3 Self-Assembling Peptides

Self-assembling peptides can also be used as a delivery system to deliver antigens to target cells. Self-assembling peptides can spontaneously form into ordered structures in response to changes in pH, solvent, co-assembling molecules, temperature and ionic strength. Th ey can have a diverse range of properties and can be manufactured to form , nanovesicles, nanofibers, nanotubes, nanoribbons and hydrogels . Self-assembling peptide deliver systems have a number of advantages over liposomes or nanoparticles including high drug loading, low drug leakage, biodegradability and are highly permeable to target cell membranes.

6.3 New Emerging Vaccine Adjuvants

As Paston et al have noted:

Other newer adjuvants are also being investigated to increase the efficacy of a cancer vaccine, these include the CD40 agonists, these directly target the antigen to the early endosomes of DCs and mediate cross presentation. Although CD40 agonist antibodies have not been extensively studied in clinical trials as a vaccine adjuvant, they have been studied independently as monotherapy. A number of preclinical mouse models have shown that CD40 agonists can be used in combination with TLR agonists in a vaccination strategy, whether this translates into clinical efficacy is still to be determined.

Another class of potential adjuvants is the Stimulator of interferon genes protein (STING) agonists. STING is a transmembrane protein located in the endoplasmic reticulum; its activation triggers a type I interferon response in response to intracellular DNA. STING agonists include synthetic cyclic dinucleotide derivatives and cyclic di-guanosine monophosphate, these have all shown anti-tumor activity in mice.

STING expression is highest on T cells and STING activation can lead to T cell apoptosis, such effects are not seen with macrophages and DCs. To use a STING agonist in a cancer vaccine it would need to be combined with an adjuvant or delivery system that targets only myeloid cells in vivo preventing T cell apoptosis. STING agonists do induce some systemic toxicity and to overcome this intratumoral injection is the preferred route of administration. In addition, preclinical studies of STING agonists have been complicated by their differential binding properties in murine and human cells.

The potential toxicity of STING agonists and lack of specific targeting could limit their use as adjuvants in a cancer vaccine. In addition to using pathogen derived molecules as adjuvants a number of cytokines have also been shown to act as an adjuvant. Immunostimulatory cytokines such as IL-2, IFN, IL-12 and granulocyte-macrophage colony stimulating factor (GM-CSF) have all been investigated, although recent studies have focused mainly on their application in cellular based therapies and vaccines. GMCSF is the most studied immunostimulatory factor and has been included in numerous cancer vaccine trials. In preclinical studies GM-CSF looked a very promising candidate, it helps recruit DCs to the injection site, it can promote the maturation of DCs and antigen presentation resulting in an enhanced adaptive immune response.

However, in clinical trials GM-CSF has generated disappointing results with only a few trials having shown a clinical benefit, the results across the majority of trials have been inconsistent. Preclinical studies indicated that GMCSF could expand MDSCs resulting in the suppression of cellmediated anti-tumor responses. The effect of GM-CSF was also observed in clinical trials where a low dose of GM-CSF induced the expansion of CD14 positive, HLA-DR low/negative myeloid cells.

In another study GM-CSF was used within complete Freund's adjuvant and resulted in a low T cell response when compared to vaccine adjuvant without GM-CSF. Despite these results a number of clinical trials are currently underway using GM-CSF as an adjuvant component.

7 CANCER OPTIONS

We will now examine several cancers that have vaccine interest. Breast cancer, BCa, appears to have been addressed with multiple paths depending on surface

7.1 BREAST

Breast cancer, BCa, is the most diagnosed cancer in women. Over the past two decades multiple surface markers and genetic changes have been determined. There are a significant set of treatments available. HER2+ BCa was once the most lethal and now appears to be treatable with increased survival even in patients with metastatic disease.

7.1.1 mAbs and Conjugates

As Pallerla et al note regarding mABs and conjugates:

Treatment of HER2-positive breast cancer with chemotherapeutic agents alone elicited a poor response. The discovery of tumor-associated antigens (TAA) has facilitated the emergence of immunotherapy. Immunotherapy with respect to cancer can be defined as the interference of the immune system for the mitigation of cancers. Monoclonal antibodies that have anti-tumor properties were developed against the HER2 receptor. The intervention of the tumor growth via monoclonal antibodies falls under the category of passive immunity. Trastuzumab was the first FDA-approved monoclonal antibody recommended for treating HER2-positive metastatic breast cancer.

It causes anti-tumor effects through various mechanisms such as induction of apoptosis, induction of cell cycle arrest, antibody-dependent cell-mediated cytotoxicity (ADCC), inhibition of HER2 extracellular domain shedding, and inhibition of downstream signal transduction pathways. Additionally, monoclonal antibodies and their conjugates such as pertuzumab, trastuzumab emtansine (T-DM1), and fam-trastuzumab deruxtecan were also approved by the FDA for treating HER2-positive breast cancer patients.

In the EMILIA study, T-DM1 exhibited improved survival for the second-line treatment of metastatic HER2-positive breast cancer compared to the existing standard therapy, capecitabine with lapatinib, a HER2 tyrosine kinase inhibitor. T-DM1, compared to trastuzumab, has also been shown to improve disease-free survival after surgery in those patients who have residual cancer after receiving neoadjuvant chemotherapy in the KATHERINE trial. Fam-trastuzumab was studied in a phase II clinical trial, which showed promising efficacy results in those patients diagnosed with metastatic HER2-positive breast cancer who failed T-DM1.

Using monoclonal antibodies for cancer therapy is an effective and efficient strategy to treat breast cancer, but it has its own drawbacks such as the cost, treatment duration and frequency, resistance, and tolerance. Furthermore, these monoclonal antibodies show temporary disease control once the tumor is metastasized; hence, there is a need for therapies that elicit anti-tumor

effects on metastatic tumors. Due to the aggressiveness of HER2-positive breast cancer, there is also a need to minimize the chance of relapse in those with a curable disease.

Despite the development of targeted therapies, tyrosine kinase inhibitors, as well as monoclonal antibodies and their toxin conjugates, all metastatic tumors develop resistance, and nearly one-third of HER2+ breast cancer patients develop resistance to all these therapies.

Thus, passive immunotherapy approaches have limitations and need continuous administration over a long period. On the other hand, a vaccine which introduces antigens acts on the cancer cells, causing prolonged activation of the immune system. Vaccines have a number of advantages compared to chemotherapy and monoclonal antibodies.

7.1.2 BCa Vaccines

As Pallerla et al note regarding possible vaccines:

Potential cancer relapse can be averted by activating long-term immunological memory with an effective vaccine that can protect against various tumor antigens.

Vaccines are not required to be administered frequently and, historically, vaccines are comparatively safer than chemotherapy. The first attempt to use a cancer vaccine was more than a century ago. In 1902, von Leyden and Blumenthal used an autologous tumor cell suspension as a vaccine and treatment for cancer patients.

During the 1950s, animal studies revealed that cancer tumors induced in mice by chemicals were immunogenic. Since then, there have been attempts to design a vaccine for cancer. Among breast cancer types, HER2- positive and triple-negative breast cancer (TNBC) subtypes are most immunogenic. Thus, for these types of cancer, activating the patient's immune system is a promising approach. Although overall progress is slow and clinical translation of this knowledge faced challenges, preclinical studies provided strong support for cancer vaccines, and there are some success stories …

Breast cancer treatment using chemotherapy, hormonal therapy, passive immunotherapy, and other modalities has made a major contribution to the treatment of breast cancer.

However, long-lasting effects are limited, and disease relapse and progression are observed in some patients. ***The discovery of breast cancer as immunogenic and the success of therapeutic vaccines such as Sipuleucel-T in treating prostate cancers raised the prospect of utilizing vaccination to manage breast cancer.*** *Several preclinical studies are ongoing, and many vaccine candidates for treating breast cancers are currently in clinical trials. Some vaccine candidates in the advanced stage of clinical trials are showing promising results in treating breast cancer.*

The vaccine candidates for managing HER2-positive breast cancers are progressing well with promising results. A single-agent E75 peptide-based vaccine candidate is being studied in a phase III clinical trial and in combination with trastuzumab in a phase II study*.*

Active immunotherapy could be an effective treatment regimen for managing breast cancer along with other therapies such as surgery, radiation, chemotherapy, endocrine therapy, and monoclonal antibodies. Active immunotherapy has the ability to produce antibodies for specific TAA, which promotes long-lasting effects.

However, until now, no therapeutic vaccines have been approved by the US FDA for treating breast cancer. The success of cancer vaccines depends on a better understanding of the tumor microenvironment, including immune-suppressing pathways and tumor-evading pathways, the discovery of specific tumor-associated antigens, effective vaccine formulations, etc. There is promising efficacy data regarding the treatment of breast cancer by designing personalized vaccines based on TTAs and genetic mutations.

In the case of personalized medicine, effective molecular stratification of breast cancer, vaccine formulation, and cost-effective vaccine manufacturing process need to be considered. In addition, clinical trials combining immunotherapy with other treatments that might produce an effective and synergic treatment regimen for breast cancer patients need to be explored.

While therapeutic cancer vaccines have shown some promise, they have not shown significant clinical benefits compared to immunotherapy such as immune checkpoint blockade. Hence, combination strategies with immune checkpoint inhibitors and antiangiogenic therapies have been proposed. Clinical trials consisting of large cohorts of patients are necessary to evaluate therapeutic efficacy of the proposed vaccine therapies.

Considering the cost of cancer drugs and the survival rate, mutation of proteins that are involved in cancer development, and resistance pathways, therapeutic vaccines have promise in the future of cancer therapy

7.2 PROSTATE

Prostate cancer, PCa is now the most common in men. It can be quite aggressive but many cases are more indolent, yet even then the metastatic state is terminal. Unlike BCa, PCa has seen fewer effective therapeutics. Surgery and radiation of various forms is the most common treatment options along with androgen deprivation therapy, ADT, after a metastatic state determined.

As Rastogi et al note the following regarding prostate cancer vaccines:

7.2.1 Non-antigen-specific vaccines

Vaccines can be broadly classified as non-antigen specific, typically whole-cell vaccines, for which the specific antigenic target is not known, or antigen specific. Whole-cell vaccine approaches initially leveraged knowledge of early microbial vaccines, in which the entire pathogen was inactivated and then readministered into the host in an attempt to generate protective immunity to one or more antigens presented by the pathogen. This approach was favoured in the absence of known tumour-specific target antigens, which are possibly different

among individuals. In early studies, irradiated prostate cancer cells, chemically coupled to rabbit γ-globulin as a foreign protein, were used as vaccines.

The use of this approach showed modest induction of tumour-associated antibodies, and further efforts focused on increasing the immunogenicity of these cellular vaccines. In a phase I/IIb trial, an autologous prostate cancer vaccine consisting of prostate cancer tissue obtained at the time of prostatectomy and treated ex vivo to upregulate major histocompatibility complex class I (MHC-I) and MHC-II expression was used to immunize patients at risk for prostate cancer recurrence30. In this study, after 5 years, a reduction in disease recurrence was observed in patients receiving the vaccine (PSA undetectable in 17 of 20 patients, 85%), compared with a non-randomized control group consisting of untreated patients (PSA undetectable in 10 of 21 patients, 48%).

7.2.2 Antigen-specific vaccines

A major disadvantage of non-antigen-specific vaccines was the absence of a defined target as a measure of immune response. Indeed, only clinical responses could be evaluated, and no known antigens were available to measure whether vaccination led to a biological effect. Additionally, this approach led to the co-administration of thousands of antigens that might be theoretically harmful, irrelevant or diminish the response to actually favourable antigens.

In the development of infectious disease vaccines, such as for hepatitis B, using vaccines focused on a particular antigen was preferable compared with using an entire virus, particularly if the virus could not be cultured. This antigen-specific vaccine led to a highly potent protective immunity and enabled measurement of whether an individual had been effectively immunized. These principles have favoured the development of antigen-specific vaccines, in most cases with the ultimate goal of developing multi-valent vaccines targeting multiple defined antigens.

7.2.3 Peptide vaccines

Several groups have identified MHC-I-restricted peptide epitopes derived from multiple prostate-specific proteins, including PSA, prostatic acid phosphatase (PAP) and PSMA.

Phase I clinical trials have been conducted with these and other peptides, in which peptides were delivered directly, with or without various adjuvants, in patients with metastatic prostate cancer. In general, results from these trials have shown no toxicity and some evidence of immune response being elicited towards the immunizing peptide but no substantial clinical benefit in terms of PSA decline, objective radiographic response or delays in disease progression.

7.2.4 Antigen-loaded DC vaccines

The ability of DCs to take up exogenous antigens and prime T cells has led to a multitude of trials in which a target antigen was directly delivered using DCs loaded with proteins, peptides or nucleic acids. In 1996, autologous DCs from patients with prostate cancer were cultured ex vivo for the first time with either autologous tumour cell lysates or HLA-A2-restricted peptide epitopes from PSMA to generate cytotoxic T lymphocytes in vitro. Subsequently, the same group

conducted trials using patient autologous DCs cultured with putative HLA-A2- restricted MHC-I epitopes from the PSMA protein.

In a phase II trial including 37 patients with prostate cancer at different stages who experienced recurrence after local therapy, the investigators reported complete response in 1 patient and partial response in 10 patients, 3 of whom had at least a 50% reduction in serum PSA68. However, in this study, Prostasin scans, which detect prostate cancer on the basis of PSMA expression, were used to identify radiographic responses. The high variability of this method, which is no longer used in clinical practice and also detects the target of the vaccine, probably confounded the interpretation of clinical response.

A randomized phase III trial to assess this approach in patients with mCRPC was planned but never completed, primarily owing to funding constraints. The company developing this vaccine opted to pursue a similar approach using autologous DCs as a vaccine but loaded ex vivo with autologous tumour lysates rather than peptides based on results from a non-randomized clinical trial in which prolonged OS (18.3 months) had been observed in patients with glioblastoma70.

In another approach, DCs isolated from patients with prostate cancer and transfected with mRNA encoding PSA were shown to stimulate a potent antigen-specific cytotoxic T cell response71. Similarly, in a small study including 13 patients with metastatic prostate cancer, autologous DCs were loaded with mRNA encoding PSA72, with the results showing a decreased PSA slope and increased PSA-specific T cell responses in all patients; unfortunately, this approach has not advanced further, for unclear reasons.

In another trial, patients with mCRPC were treated with autologous DCs that had been cultured ex vivo with the mouse homologue of PAP, in principle using the xenoantigen form of the protein to circumvent immune tolerance to the native antigen. T cell immune responses to the native protein were elicited by this approach, and 6 of 21 patients had stable disease following treatment.

7.2.5 Viral and bacterial vaccines

A major function of the immune system is to recognize and respond to microbial infections. This feature has led to the engineering of viral and bacterial vectors to encode tumour-associated antigens as a tool to elicit antitumour immunity. Perhaps the best-studied approach has been the one using vaccinia and other poxvirus vectors. Vaccinia virus is a DNA orthopoxvirus that replicates in the cytoplasm. Delivery of poxvirus vectors results in infected cells expressing peptides from virally derived proteins in MHC-I molecules, stimulating a vigorous cell-mediated response against the antigenic proteins. Additionally, vaccinia virus has a large genome and, therefore, is an ideal vector for deliveries in which transduction of multiple genes or genes encoding large proteins are involved

7.2.6 Nucleic acid vaccines

Nucleic acid vaccines, in which plasmid DNA or mRNA are used to encode defined target antigens, are similar to viral vector vaccines in terms of mechanism of action but have the

advantage of not expressing any foreign viral genes. Thus, nucleic acid vaccines are safer than viral and bacterial vaccines, have a low likelihood of genome integration, and do not elicit an immune response towards the vectors. Plasmid DNA derived from bacterial DNA encodes an antigen but might also provide TLR9 agonist signals and activate other cytoplasmic DNA sensing molecules such as AIM2, RIG-I and STING125; similarly, RNA might activate other TLR sensors such as TLR3 and TLR7.

The activation of different innate-sensing pathways through these approaches could potentially lead to different immune responses. Currently, much enthusiasm exists around the development of mRNA vaccines as cancer vaccines considering the success of this vaccine type against SARS-CoV-2; however, little comparison between DNA versus RNA vaccines in terms of potential differences in immunogenicity or antitumour efficacy exists. Conceptually, RNA vaccines have a potential advantage over plasmid DNA vaccines as mRNA does not need to pass the nuclear membrane for transcription to take place within transfected cells and, therefore, higher amounts of protein can be rapidly generated from transfected mRNA than those obtained with DNA. However, RNA is less stable than DNA owing to tissue RNases. Thus, historically, DNA vaccines have been explored the most.

7.2.7 Nucleic acid vaccines — mRNA.

To date, only one vaccine approach using mRNA through direct injection has been reported. In a phase I/IIa study, an mRNA vaccine, known as CV9103, encoding PSA, prostate stem cell antigen, PSMA and six-transmembrane epithelial antigen of the prostate 1 (STEAP1) was given by intradermal injection to 76 patients with CRPC155. A total of 26 of 33 evaluable patients developed an immune response to one or more antigens, and 15 of 33 patients developed an immune response to multiple antigens. Based on this evidence of immunogenicity, and on the observation that the estimated OS of patients with metastatic disease was favourable at 31.4 months, the vaccine was modified to include two additional antigens, PAP and MUC1, for further clinical evaluation.

7.3 PANCREAS

Pancreatic cancer is one of the most aggressive cancers in humans. It is a silent killer since generally there are no symptoms until significant metastasis has occurred and then survival is months at best. The incidence is low but mortality high. As Rojas et al note the results of a combine therapy targeting some 20 antigens:

Pancreatic ductal adenocarcinoma (PDAC) is lethal in 88% of patients1, yet harbours mutation-derived T cell neoantigens that are suitable for vaccines.

Here in a phase I trial of adjuvant autogene cevumeran, an individualized neoantigen vaccine based on uridine mRNA–lipoplex nanoparticles, we synthesized mRNA neoantigen vaccines in real time from surgically resected PDAC tumours.

*After surgery, we sequentially administered atezolizumab (**an anti-PD-L1 immunotherapy**), autogene cevumeran (**a maximum of 20 neoantigens per patient**) and a modified version of a*

*four-drug chemotherapy regimen (**mFOLFIRINOX, comprising folinic acid, fluorouracil, irinotecan and oxaliplatin**).*

The end points included vaccine-induced neoantigen-specific T cells by highthreshold assays, 18-month recurrence-free survival and oncologic feasibility. We treated 16 patients with atezolizumab and autogene cevumeran, then 15 patients with mFOLFIRINOX. Autogene cevumeran was administered within 3 days of benchmarked times, was tolerable and induced de novo high-magnitude neoantigen-specific T cells in 8 out of 16 patients, with half targeting more than one vaccine neoantigen.

Using a new mathematical strategy to track T cell clones (CloneTrack) and functional assays, we found that vaccine-expanded T cells comprised up to 10% of all blood T cells, re-expanded with a vaccine booster and included long-lived polyfunctional neoantigen-specific effector CD8+ T cells. At 18-month median follow-up, patients with vaccine-expanded T cells (responders) had a longer median recurrence-free survival (not reached) compared with patients without vaccine-expanded T cells (non-responders; 13.4 months, P = 0.003). Differences in the immune fitness of the patients did not confound this correlation, as responders and non-responders mounted equivalent immunity to a concurrent unrelated mRNA vaccine against SARS-CoV-2.

Thus, adjuvant atezolizumab, autogene cevumeran and mFOLFIRINOX induces substantial T cell activity that may correlate with delayed PDAC recurrence.

The above result appears to be an attractive first step in managing an extremely aggressive cancer. We continue to detail some of the results.

7.3.1 Current Approaches

As Huff and Zaidi have noted:

The development of a type of immunotherapy that uses drugs known as immune-checkpoint inhibitors (ICIs) has revolutionized cancer treatment. ICIs act by helping to unleash a person's immune system against mutated versions of proteins (termed neoantigens) that are expressed solely by cancer cells.

Peptide segments of neoantigens are likely to be viewed as foreign by the immune system, and can activate immune cells called T cells (specifically, those known as CD8 T cells), which are capable of killing cancer cells. Pancreatic cancers generally don't respond to ICIs. This is thought to be partly because these tumours express lower levels of neoantigens than do other types of tumour, and thus are less likely to activate a strong immune response from antitumour T cells. Rojas et al. 1 challenge this idea on page 144, and describe an approach in which neoantigen-specific T cells can be activated — by a vaccine that encodes neoantigens specific to the individual. Their study builds on previous work showing that individuals who are long-term survivors of pancreatic cancer have high-quality neoantigens that can stimulate antitumour T cells.

Rojas and colleagues designed messenger RNA vaccines (Fig. 1) corresponding to neoantigens for 16 people with pancreatic cancer whose tumours had been surgically removed. Individuals who undergo such surgery generally have up to an 80% chance of disease recurrence4. The mRNA vaccines encoded a maximum of 20 neoantigens per patient, identified by sequencing DNA and RNA from the patients' surgically removed tumours. Vaccines were given intravenously around nine weeks after surgery, with plans to speed up the time for vaccine generation and administration in the next stage of the study (a phase II clinical trial).

This rapid time to treatment underscores the benefit of mRNA-based cancer vaccines, particularly for highly aggressive tumours. T cells that recognized specific neoantigens corresponding to the mRNA-encoded peptides were detected in the blood after vaccination in half of the people in the trial — these individuals were termed immune responders. Of these responders, half had T-cell responses to more than one neoantigen (a polytopic response), whereas the other half generated a response to a single neoantigen (a monotopic response). Remarkably, in all immune responders, there was no evidence of cancer recurrence at a median follow-up time of 18 months after surgery, compared with a median time to recurrence of 13.4 months in non-responders. These data are exceedingly promising, and will provide the framework for a planned further clinical trial. All patients also received a single dose of an ICI called atezolizumab before being given the mRNA vaccine.

Atezolizumab targets the protein PD-L1 found on tumour cells, and acts by reinvigorating pre-existing tumour-reactive T cells that have entered a dysfunctional, 'exhausted' state because of interactions between PD-L1 and the immunosuppressive receptor PD-1. The authors analysed patient blood samples after ICI treatment, and identified T-cell lineages that had proliferated (expanded) — a sign of T-cell activation in response to neoantigen recognition. The authors identified these lineages by sequencing DNA corresponding to part of the T cell involved in immune responses (the T-cell-receptor β-chain).

The T cells that proliferated with atezolizumab treatment were different from those that proliferated after mRNA vaccination, providing evidence that the vaccine had activated neoantigen-specific T cells. Four weeks after the final mRNA vaccination, patients received chemotherapy, which can sometimes suppress immune cells, but the authors found that the vaccine-boosted T cells were not suppressed. This highlights the fact that sequential combination treatment strategies are feasible for people with pancreatic cancer.

7.3.2 mRNA Vaccine

As Rojas et al note:

We demonstrated that adjuvant autogene cevumeran[25], an individualized neoantigen vaccine based on uridine mRNA–lipoplex nanoparticles, in combination with atezolizumab and

[25] See https://ncit.nci.nih.gov/ncitbrowser/ConceptReport.jsp?dictionary=NCI%20Thesaurus&code=C175745 *Autogene Cevumeran, An mRNA-based individualized, therapeutic cancer vaccine targeting an unspecified amount of tumor-associated antigens (TAAs) that are specifically expressed in the patient's cancer, with potential immunostimulatory and antineoplastic activities.*

mFOLFIRINOX, is safe, feasible and generates substantial neoantigen-specific T cells in 50% of unselected patients with resectable PDAC. Vaccine-expanded T cells were durable, persisting up to 2 years despite post-vaccination mFOLFIRINOX treatment.

High-magnitude vaccine-induced T cell responses, the focus of our immune response analysis that included a new method to track vaccine-expanded clones, correlated with delayed PDAC recurrence.

Despite the limited sample size, these early results warrant larger studies of individualized mRNA neoantigen vaccines in PDAC. As multiple immunotherapies31 have emerged for immune-inflamed tumours, there remains a need for new immunotherapies for the majority of patients with non-inflamed tumours that are largely insensitive to current immunotherapies.

Indeed, the prevailing thought has been that the low passenger mutation rate of such tumours renders them with insufficient neoantigens for vaccines. Here, we provided evidence that despite the low mutation rate of PDAC, a mRNA vaccine can induce T cell activity against neoantigens in this cancer, a non-inflamed tumour with predominantly immune-excluded or desert phenotypes.

Whether mRNA neoantigen vaccines can similarly activate T cells in other non-inflamed cancers should be more broadly tested. We did not find evidence that the correlation of vaccine response to delayed recurrence is confounded by known prognostic variables, such as lymph node or margin-positive disease.

Non-responders on average had slightly larger primary tumours than responders; however, larger primary tumour size did not correlate with shorter RFS. As the uridine mRNA–lipoplex vaccine technology is based on potent antigen delivery into lymphoid compartments and stimulates weak T cell responses in splenectomized mice20, it is notable that non-responders were also marginally enriched in patients with splenectomies (Extended Data Fig. 1b). Furthermore, that vaccines induced high-magnitude T cell responses in 50% of patients may highlight the need for biomarkers to select optimal patients and tumours for this treatment.

Of note, although autogene cevumeran is designed to activate both neoantigen-specific CD4+ and CD8+ T cells and we find it activates high magnitude CD8+ T cells in PDAC, the primary and confirmatory immune response assays in this study do not distinguish CD8+ from CD4+ T cell responses. In fact, as these assays bias towards high-magnitude T cell responses, assays that detect lower magnitude responses may include both CD4+ T cell responses and pre-existing responses.

Upon administration, autogene cevumeran is taken up and translated by antigen presenting cells (APCs) and the expressed protein is presented via major histocompatibility complex (MHC) molecules on the surface of the APCs. This leads to an induction of both cytotoxic T-lymphocyte (CTL)- and memory T-cell-dependent immune responses against cancer cells expressing the TAA(s).

8 OBSERVATIONS

The previous discussions were predicated on the most recent concerning cancer vaccines. It seems clear that this is still at an early stage.

8.1 T CELL EXHAUSTION

As we noted herein, T cell exhaustion is a well-known phenomenon. Vaccines currently in use all too often have a limited lifetime. That is part of the exhaustion process. However just what causes this exhaustion is not yet fully understood, and subsequently there is no mitigating factor. As such one may wonder if a vaccine is no better than polyspecific conjugate antibodies.

8.2 SIZE MATTERS

One of the difficulties in presenting many of these constructs is the simplicity of the graphics. In reality the malignant cells, and all other cells for that matter, are essentially "fur balls" of surface protein arguably arranged in no specific manner. Proximity of ligands or receptors is uncertain and this fact itself may effect cell responses. T cell when ready to attack roll over and under other cells looking for responses. The question is; does the size of the cell and its receptors and ligands across the surface matter.

For example, a neutrophil is about 300 μm^3 or about 7 μm in radius, or 14 μm in diameter[26]. A hart cell is 15,000 μm^3 and thus 48 μm in diameter. Now a base pair is about 0.34 μm long and a surface protein segment may occupy 0.09 μm^2. But the surface area of a typical cell, say of 15 μm is ($4\pi r^2$) or 2826 μm^2. Thus, meaning that fully packed we could have almost 29,000 surface proteins. More than likely, we can expect a small percentage covered. But one must ask if there can be interfering proteins, blocking connections. Consider the simple example of a TCR and PD-1 presence, just what are the physical limit?

8.3 NEO-ANTIGENS AND STEM CELLS

Neo antigens are the Ag we find on the cancer cells. If one accepts a modicum of stem cells behavior then should we not look for the stem cell and disregard others. If so how do we identify the stem cells.

8.4 HOW CLOSE IS CLOSE

As we noted above proximity is important. As Warboys and Davis have recently noted:

Chimeric versions of PD-1 that contained different numbers of Ig domains in its extracellular tail had differing inhibitory potential, consistent with the kinetic-segregation model of positioning proteins at the immune synapse according to their size. PD-1 with large extracellular domains were excluded from TCR clusters and could not prevent downstream TCR signalling

[26] See Milo et al also https://www.nature.com/scitable/topicpage/dna-packaging-nucleosomes-and-chromatin-310/ also http://cyberbridge.mcb.harvard.edu/dna_2.html

and IL-2 secretion. Additionally, PD-1 phosphorylation only occurred when PD-1 was ligated and colocalized with the TCR, which correlated with SHP-2 recruitment. This is evidence that PD-1 proximity to the TCR is critical to initiate functional inhibitory signalling.

This is not limited to T cells, as inhibition by Killer Ig-like receptors required proximity to the activating receptor NKG2D at the surface of human NK cells, which could also be perturbed by altered protein size. Other evidence is that TCR stimulation leads to an accumulation of CTLA-4 at the immune synapse in a manner dependent on the TCR signalling strength.

The Src-family kinase, Lck, can phosphorylate cytoplasmic CTLA-4 tyrosine residues which promotes its localisation from subcellular vesicles to the membrane. Stimulation of the TCR generates hubs of Lck activity at the immune synapse that could lead to localised surface enrichment of CTLA-4 where it can bind to B7 ligands to provide negative feedback. ... Providing inhibitory signals to cells that do not require inhibition would feasibly be wasteful of cellular activity and resources. This could provide an evolutionary rationale for inhibitory signalling to only act where and when it is necessary. Often, textbook diagrams depict receptor transduction occurring solely upon ligation, but this is too simplistic as inhibitory receptor signalling is context specific. Initiating inhibitory processes likely requires signals from local stimulatory receptor signalling hubs, as is the case for PD-1, CTLA-4 and potentially for TIGIT.

In some cases, inhibitory receptors may not require signalling to function as their proximity to stimulatory receptors by itself can be inhibitory, as with LAG3. Limiting the ability of inhibitory receptors to function at precise nanoscale locations of stimulation permits a spatiotemporal regulation governed by stimulatory signals, providing highly efficient regulatory mechanisms.

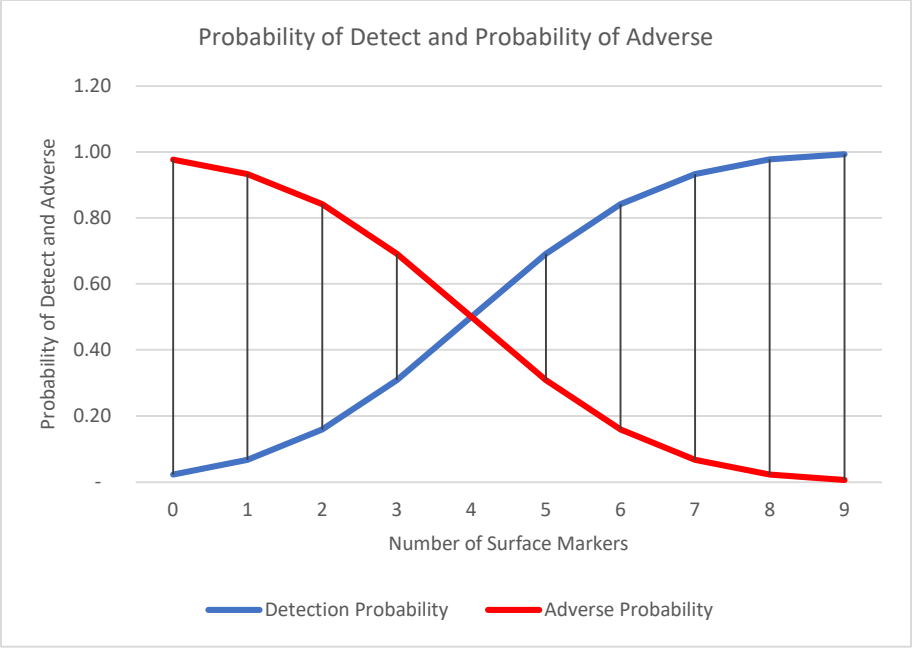
8.5 HIGH MUTATION RATES

We have seen in the COVID pandemic a virus with a significant mutation rate. One could likewise ask what is the mutation rate of cancer cells, especially stem cells.

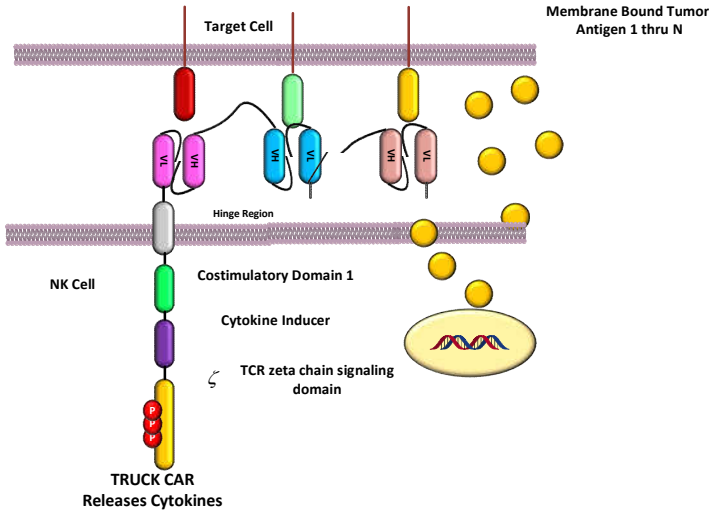
8.6 POLYSPECIFIC-CONJUGATES VS CANCER VACCINES (MULTI ANTIGEN TARGETING)

Polyspecific antibodies, polyAb, integrated with a cell killing therapeutic, creates a conjugate. The polyAb can target specific sets of surface Ag, many actually, thus allowing targeting of the specific cancer cells. The therapeutic then destroys the cell.

Now the basic principle we espouse is that if we demand a set of multiple targets s shown above, not just one, then we get better targeting and less bad consequences. Namely we get to eliminate just what we want. We show that graphically below:

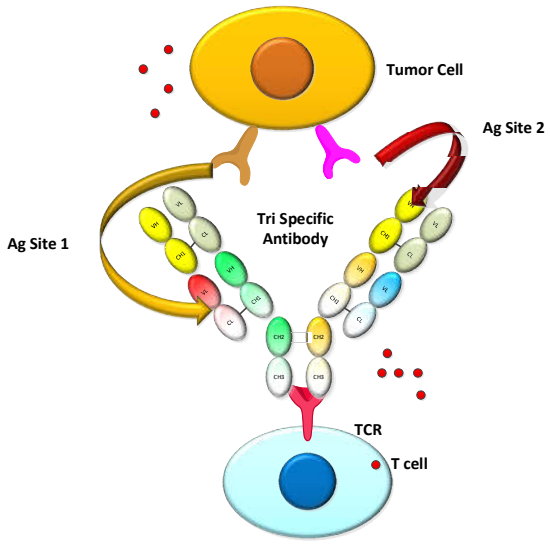


Now we can use what we call polyspecific Ab as shown below:

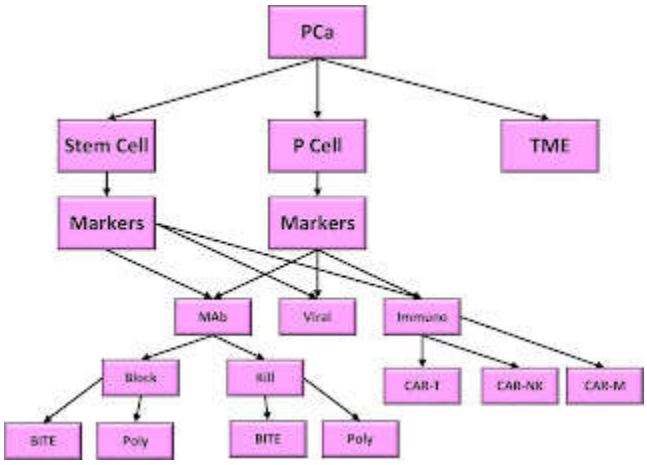


This is a 4th generation CAR. We have three ligand attachment areas plus when all 3 are attached they activate the release of cytokines and kill the cancer cell. These types of poly Ab are relatively easy to make and if they do the full attachment their have limited morbidity. They go after just the cancer cells and no others.

Now we can use Ab alone. Below we show a poly Ab attaching to a cell and then activating an immune cell.



These approaches are all based upon existing protocols. The demand the ability to identify the cancer cell and then produce the CAR or Ab as noted.



8.7 Multi-Antigen Identification

How does one determine multi-antigens? This is a complex issue. Ag result from mutations. But mutations may no transcribe nor may they translate. If they do they may not become surface markers. As Leko and Rosenberg have noted

Identification of specific tumor antigens, as well as T cells that recognize them, is essential for the design and execution of both vaccine- and ACT-based immunotherapy approaches. Regardless of which of the methods is used, immunogenicity of each newly discovered antigen needs to be validated in functional assays.

This is accomplished by demonstrating that T cell activation occurs only upon encountering a specific epitope, but not the corresponding control (e.g., wild-type peptide for mutant antigens)

that is bound to the same MHC molecule. A mere finding that a peptide is bound or even predicted to bind to an MHC molecule expressed by cancer is not a proof of its immunogenicity and can be misleading.

8.7.1 cDNA Expression Library Screening

This method was the first one to be used for tumor antigen.

1. ***It typically starts with isolation of a tumor-reactive T cell population, either from a patient's tumor or from the PBL.***

2. ***Next, total RNA is isolated from tumor cells and converted into pools of cDNA plasmids, which are transfected into recipient cells (such as COS-7 or 293-HEK cells), often together with plasmids encoding specific MHC molecules.***

3. ***T cells are then co-cultured with the transfected cells, and assayed for recognition of cDNA pools and, subsequently, individual cDNA plasmids.***

4. ***Those that elicit recognition by T cells are used to define the encoded epitopes (peptides), which are then synthesized, pulsed onto the MHC-transfected cells, and validated in a reaction with the same T cell population.***

Although it allows identification of most tumor antigen types, this method is laborious and time consuming, and therefore inappropriate for high-throughput antigen screening.

Furthermore, due to difficulties in cloning of GC-rich sequences and large or poorly expressed RNA transcripts, it may be insufficiently sensitive to detect some types of mutated tumor antigens.

8.7.2 Next-Generation Sequencing-Based Screening Methods

Autologous T cells can be screened using peptides that arise from various types of cellular proteins, including those that harbor tumor-specific mutations.

The sequences of these peptides can be determined by interrogating tumor and normal DNA or RNA by nextgeneration sequencing (NGS) methods. To facilitate screening, especially in malignancies that harbor a vast number of mutations, such as melanoma, candidate peptides can be filtered by using algorithms that predict their immunogenicity or by directly assessing their presentation on the tumor cell surface using immunopeptidomics.

8.7.3 Prediction Algorithm-Based Screening Methods—

Various algorithms have been developed to predict whether peptides derived from a specified protein, either wild-type or mutant, are available to interact with TCRs on T cells.

This is most commonly done by predicting their ability to bind to specific MHC molecules that are expressed by the cancer, as exemplified by various iterations of the NetMHCpan algorithm.

These and other algorithms have been trained on data resulting from in vitro binding assays involving peptides with defined amino acid sequences, or on data obtained by immunopeptidomics.

1. ***To obtain binding predictions, researchers input protein or peptide sequences, specify the desired peptide length, and select an MHC molecule of choice.***

2. ***The algorithms then generate a list of possible resulting peptides ranked by their MHC-binding affinities, which are usually expressed as either the half maximal inhibitory concentration (IC50) or as a percentile rank.***

3. ***Peptides passing a certain consensus threshold (i.e., <500 nM or ≤2, respectively) are generally considered MHC binders and are selected for antigen screening.***

In one such screening approach, whole-exome sequencing (WES) data from matched tumor and normal DNA is coupled with translation in silico to identify peptides containing tumorspecific non-synonymous mutations. A portion of peptides that is predicted to strongly bind to patients' own MHC class I molecules is then synthesized, pulsed onto the APCs, and tested for recognition by the autologous CD8+ T lymphocytes.

In a variation of this approach, peptide-pulsed APCs are replaced with artificial multimeric peptide-MHC complexes (e.g., MHC tetramers), which are generated by joining a variable number of fluorescently labeled or genetically barcoded MHC molecules and loading them In one such screening approach, whole-exome sequencing (WES) data from matched tumor and normal DNA is coupled with translation in silico to identify peptides containing tumor specific non-synonymous mutations.

A portion of peptides that is predicted to strongly bind to patients' own MHC class I molecules is then synthesized, pulsed onto the APCs, and tested for recognition by the autologous CD8+ T lymphocytes.

In a variation of this approach, peptide-pulsed APCs are replaced with artificial multimeric peptide-MHC complexes (e.g., MHC tetramers), which are generated by joining a variable number of fluorescently labeled or genetically barcoded MHC molecules and loading them with candidate peptides. These complexes can bind to complementary TCRs and thus enable quantification of T cells that recognize candidate antigens.

This approach can be efficient for identifying epitopes that are predicted to bind to prevalent MHC class I molecules, but it is still of limited use for identification of those that bind to class II or rarer class I MHC molecules.

Furthermore, as it entails testing peptide libraries tied to selected MHC molecules, this method generally fails to assess all the potential antigens expressed by the cancer. Prediction-based screening methods have several important limitations, and only a few of the predicted peptides are found to be immunogenic in validation experiments.

These limitations include suboptimal algorithm performance with less common class I and most class II MHC molecules, lack of ability to identify post-translationally modified or spliced peptides, and propensity to miss some de facto immunogenic peptides. To overcome some of these limitations, various bioinformatical approaches also incorporate algorithms that predict other protein/peptide characteristics implicated in immunogenicity.

For instance, a model with an increased ability to predict immunogenic mutated peptides has recently been developed by combining predictions of peptide-MHC binding affinity, wildtype-over-mutant affinity ratios and the stability of given peptide-MHC complexes, together with data on the expression of cognate genes (Gartner et al., unpublished data).

8.7.4 Unbiased Tumor Antigen Screening—

To bypass the limitations of prediction algorithms, another WES-based approach has been developed to enable unbiased screening of all candidate antigens; i.e., without restricting the analysis to specific MHC molecules.

In this approach, metastatic tumors are surgically removed and used both to generate TIL cultures and to perform WES to identify tumor-specific non-synonymous mutations, namely single-nucleotide variants (SNVs) and small (<50 bp) insertion and deletions (INDELs). These sequences are used as templates to synthesize two types of screening libraries.

One type is prepared by synthesizing and pooling 25-mer peptides harboring a tumor-specific mutation in their center.

The second type is prepared by designing minigenes that represent the mutant 25-mers and concatenating them into tandem minigenes (TMGs), which are ultimately transcribed into RNA in vitro.

Next, autologous APCs are pulsed with peptide pools or electroporated with TMGs, allowing processing and presentation of candidate antigens on all possible autologous MHC molecules, and then cocultured with a panel of TILs.

Peptide pools or TMGs that elicit T cell activation are further deconvoluted to identify specific tumor antigens. As described in the following sections, this approach has been used to identify a number of tumor antigens arising from missense SNVs (mSNVs) or INDELs. However, it does not allow detection of antigens that arise from unmutated genes, gene fusions (some bioinformatic approaches can still enable this, but with limitations), or from aberrant RNA processing or translation.

These limitations could be overcome by utilization of RNA sequencing or whole-genome sequencing (WGS) in a similar paired tumor-vs-normal fashion.

8.7.5 Immunopeptidomics

Finally, tumor antigens can be identified by direct interrogation of the tumor immunopeptidome; i.e., all endogenous peptides that are presented by MHC molecules on the cell surface.

In this approach, after extraction from tumor cells, peptides are eluted from their complexes with MHC molecules and then subjected to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

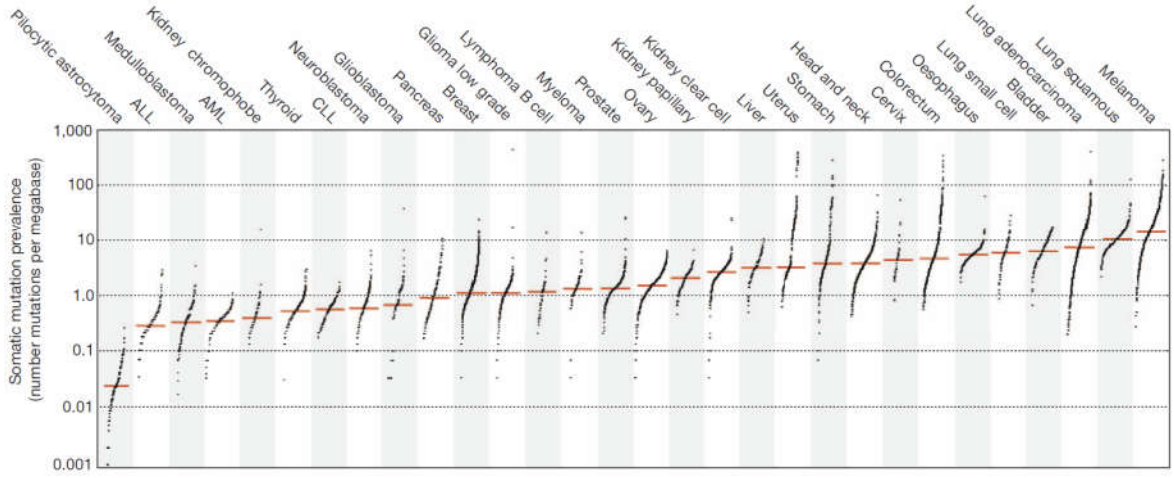
Next, in order to identify tumor-specific peptides, MS spectra are compared with customized databases, which are generated by combining NGS data from patients' tumors with the reference protein sequences.

Although this approach has the potential to uncover all of the possible classes of tumor antigens, including products of post-translational modifications that could be missed by the aforementioned sequencing approaches, its use is still limited by overall low sensitivity, especially for MHC class II-bound peptides.

Overall these methods provide some useable Ag but at an extremely high cost as well as gross lack of specificity even if done on a cell by cell basis.

8.8 Mutation Rates Count?

From Alexandrov et al we have the classic diagram of mutations by malignancy. One suspects that this is a moving target. The more mutations the more Ags that may be available and thus the increased specificity for multi-Ag targeting.



8.9 MULTI-THERAPEUTICS WORK?

As we have noted herein and in many other clinical settings the use of multi-therapeutics seems to have substantially increases efficacy. PD-1 blockers along with a vaccine plus even classic chemotherapy seems to work quite well. Given the number of dimensions however it may take a while to adapt to an optimum protocol. In fact, it may result in highly individualized treatments.

8.10 MENTAL MODELS VS REALITY

In a recent book by Gutfreund and Renn, the authros examine Einstein's thought regarding reality and models[27]. Simply stated Einstein used models only later to be verified by reality. Much of what we do herein is based on models, paradigms if you will. Reality all too often is overly complex so we assemble models that get us say 90% there. Yet cancer resides in the 10% of reality our models fail to include. Thus it is essential that we go back from time to time and question the "model world" of our presentation. Hopefully we have gotten the 90% and strive to include the 10%.

8.11 SOMETIMES IT JUST DOES NOT WORK?

Cancer therapeutics have a spotty history. What one would expect to work may not in the long run. A recent article by Mellgard et al lists a long collection of such withdrawals after a wider application. Most were early approvals. The authors conclude:

the withdrawal of agents or indications presents a diverse portrait in drug development, and in no way undermines the AA or CMA pathways. Although many clinicians have blamed surrogate endpoints for the withdrawals, our assessment shows that recent withdrawals of oncologic agents had little to do with the failure of surrogates, and one can argue that the AAs/CMAs were generally correct for the approved indications.

Instead, causes of withdrawals are usually more nuanced. Both the FDA's AA and EMA's CMA paradigms anticipate that a fraction of approvals will be withdrawn, hence the existence of policies for withdrawal and the stated need for confirmatory trials.

In exchange for bringing likely effective therapies to patients sooner, the FDA and EMA accept this risk, but do so with the expectation that everything possible will be done to properly assess efficacy and minimize risk. In this regard, these processes have fared well, and, apart from the PI3K inhibitors, it is difficult to argue that harm has resulted.

[27] See Gutfreund and Renn, The Einsteinian Revolution, Princeton, 2024,

9 REFERENCES

1. Aaronson, Growth Factors and Cancer, Science, Vol 254 Nov 1991.

2. Abbas et al, Cellular And Molecular Immunology, 10th Ed, Elsevier, 2022

3. Abdel-Azim et al, Strategies to generate functionally normal neutrophils to reduce infection and infection-related mortality in cancer chemotherapy, Pharmacol Ther. 2019 December; 204

4. Acevedo et al, Paths of FGFR driven tumorigenesis, Cell Cycle, 15 Feb 2009

5. Ahn, K., Transcription Factor NF-kB, Ann NY Aca Sci, V 1056, 2005, pp 218-233.

6. Ailles, L., I. Weissman, Cancer Stem Cells in Solid Tumors, Curr Opp in Biotech, 2007, pp 460-466.

7. Akeson and Malemud, A Role for Soluble IL-6 Receptor in Osteoarthritis, Funct Morphol Kinesiol. 2017

8. Alexandrov et al, Signatures of mutational processes in human cancer, Nature 2013

9. Allis, C., et al, Epigenetics, CSH (Cold Spring Harbor) 2007.

10. Alsaab et al, PD-1 and PDL-1 checkpoint signalling inhibition for cancer immunotherapy: Mechanism, combinations, and clinical outcomes, Frontiers in Pharmacology, 2017

11. Amulic et al, Cell-Cycle Proteins Control Production of Neutrophil Extracellular Traps, Developmental Cell 43, 449–462, November 20, 2017

12. Anderson and Simon, The tumor microenvironment, Current Biology 30, R905–R931, August 17, 2020

13. Appasani, K., MicroRNAs, Cambridge (New York) 2008.

14. Araud, T., Overview of the miRNA Pathways, Thesis, U Geneva, 2008.

15. Arneth, Tumor Microenvironment, Medicina, 2020, 56, 15

16. Badrinath et al, A vaccine targeting resistant tumours by dual T cell plus NK cell attack, Nature, 2022

17. Bajrami et al, G-CSF maintains controlled neutrophil mobilization during acute inflammation by negatively regulating CXCR2 signaling, J. Exp. Med. 2016 Vol. 213 No. 10

18. Baldo, P., et al, mTOR Pathway and mTOR inhibitors as Agents for Cancer Therapy, 2008, Curr Can Drug Targets, pp 647-668.

19. Bao et al, TGF-β2 induces proliferation and inhibits apoptosis of human Tenon capsule fibroblast by miR-26 and its targeting of CTGF, Biomedicine & Pharmacotherapy, Volume 104, August 2018, Pages 558-565, open access

20. Bart et al, Macrophage reprogramming therapy, Immunology, 2021

21. Bartel, D., MicroRNAs: Genomics, Biogenesis, Mechanism, and Function, Cell 2004 pp 281-297.

22. Baylin, S., Understanding DNA Methylation and Epigenetic Gene Silencing in Cancer, 2004 Current Therapeutics Inc.

23. Beekman, J., P. Coffer, The ins and outs of syntenin, a multifunctional intracellular adaptor protein, Journal of Cell Science 121, 1349-1355 Published by The Company of Biologists 2008.

24. Bejarano et al, Therapeutic Targeting of the Tumor Microenvironment, AACR Cancer Discovery, April 2021

25. Bercovivi et al, The Remarkable Plasticity of Macrophages: A Chance to Fight Cancer, Front in Immunology, July 2019

26. Berridge, Cell Signalling Pathways, Cell Signalling Biology, Module 2, BJ Signal, 2012

27. Bettini and Bettini, Understanding Autoimmune Diabetes through the Prism of the Tri-Molecular Complex, Frontiers in Endocrinology, December 2017

28. Biffi and Tuveson, Deciphering cancer fibroblasts, J. Exp. Med. 2018 Vol. 215 No. 12 2967–2968

29. Bilen et al, The prognostic and predictive impact of inflammatory biomarkers in patients who have advanced-stage cancer treated with immunotherapy, Cancer. 2019 Jan 1;125:127-134

30. Blanter et al, Studying Neutrophil Function in vitro: Cell Models and Environmental Factors, Journal of Inflammation Research 2021:1

31. Botehlo et al, Vascular Endothelial Growth Factor (VEGF) and Prostate Pathology, International Braz J Urol Vol. 36 : 430-438, July - August, 2010

32. Boukerche H., et al., Src kinase activation is mandatory for MDA-9/syntenin-mediated activation of nuclear factor-κB, Oncogene. 29:3054-66, 2010 May 27.

33. Boukerche, H. et al, mda-9/Syntenin: A Positive Regulator of Melanoma Metastasis, Cancer Res 2005; 65:10901-10911. Published online December 1, 2005

34. Boumber, Y., et al, Epigenetics in Cancer: What's the Future?, ONCOLOGY. Vol. 25 No. 3 March 16, 2011

35. Brena, R., et al, Mining Methylation for Early Detection of Common Cancers, PLOS Medicine December 2006, Volume 3, Issue 12|.

36. Brooks, J., et al, CG Island Methylation Changes Near the GSTP1 Gene in Prostatic Intraepithelial Neoplasia, Can Epid 1998, pp 531-536.Donkena, K., et al, Oxidative Stress and DNA Methylation in Prostate Cancer, Ob & Gyn Internat 2010, pp .

37. Brower, V., Unravelling the cancer code, Nature, March, 2011.

38. Bunz, F., Principles of Cancer Genetics, Springer(New York) 2008.

39. Buonaguro and Tagliamonte, Selecting Target Antigens For Cancer, Vaccines, 2020

40. Cai et al, Tumor-Associated Macrophages Derived TGF-β Induced Epithelial to Mesenchymal Transition in Colorectal Cancer Cells through Smad2,3-4/Snail Signaling Pathway, Cancer Res Treat. 2019;51:252-266

41. Calin and Croce, Chromosomal Rearrangements and Micro RNAs, Jrl Clin Inv 2007 pp 2059-2066.

42. Campbell, A., L. Heyer, Genomics, Proteomics, and Bioinformatics, Benjamin Cummings (New York) 2003.

43. Cantley et al, Signal Transduction, CSHL Books, 2014

44. Carpenter and Cohen, Epidermal Growth Factor, The Journal Of Biological Chemistry, Vol. 265, No. 14, Issue of May 15, pp. 7709-7712.1990

45. Carrecedo, A., et al, PTEN Level in Tumor Suppression, How Much is Too Little?, Cancer Res; 71; pp. 629–33. 2011.

46. Causton, H. et al, Microarray Gene Expression and Analysis, Blackwell (Malden, MA) 2003.

47. Challen et al, Mouse Hematopoietic Stem Cell Identification And Analysis, Cytometry A. 2009 January; 75: 14–24. doi:10.1002/cyto.a.20674.

48. Chaney et al, The Involvement of Neutrophils in the Pathophysiology and Treatment of Osteoarthritis, Biomedicines 2022, 10, 1604.

49. Chanmee et al, Tumor-Associated Macrophages as Major Players in the Tumor Microenvironment, Cancers 2014, 6, 1670-1690

50. Cheah et al, CD14-expressing cancer cells establish the inflammatory and proliferative tumor microenvironment in bladder cancer, PNAS, April 14, 2015, vol. 112, no. 15, 4725–4730

51. Chen et al, PDGF-D promotes cell growth, aggressiveness, angiogenesis and EMT transformation of colorectal cancer by activation of Notch1/Twist1 pathway, Oncotarget, 2017, Vol. 8, (No. 6), pp: 9961-9973

52. Cheng et al, Prevalence of Diabetes by Race and Ethnicity in the United States, 2011-2016, JAMA, 2019;322:2389-2398

53. Chevier et al, An Immune Atlas of Clear Cell Renal Cell Carcinoma, Cell 169, 736–749, May 4, 2017

54. Chotinantakul and Leeanansaksiri, Hematopoietic Stem Cell Development, Niches, and Signaling Pathways, Hindawi Publishing Corporation, Bone Marrow Research, Volume 2012, Article ID 270425

55. Chow, L. et al, PTEN Function in Normal and Neoplastic Growth, Cancer Ltr, V 241, 2006, pp 184-196.

56. Christopher et al, Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice, J. Exp. Med. Vol. 208 No. 2, 251-260, 2011

57. Cirri et al, Cancer associated fibroblasts: the dark side of the coin, Am J Cancer Res 2011;1:482-497

58. Clarke, Normal Bone Anatomy and Physiology, Clin J Am Soc Nephrol 3: S131–S139, 2008

59. Com et al, Targeting Fibroblast Growth Factor Pathways in Prostate Cancer, Clin Cancer Res; 19 November 1, 2013

60. Cortez-Retamozo et al, Origins of tumor-associated macrophages and neutrophils, PNAS, February 14, 2012, vol. 109, no. 7

61. Costa, F., et al, Concise Review: Cancer Testis Antigens, Stem Cells and Cancer, Stem Cells 2007 pp 707-711.

62. Croce, C., Causes and consequences of microRNA dysregulation in cancer, Nature RE Reviews, Genetics, 704, October 2009, VOL 10.

63. Curiel (ed.), Cancer Immunotherapy, Springer Science and Business Media, New York, 2013

64. Dahia, P., PTEN, A Unique Tumor Suppressor Gene, Endo Rel Can, V 7 2000, pp 115-129.

65. Dahlman, A., et al, Effect of Androgen Deprivation Therapy on the Expression of Prostate Cancer Biomarkers, Pros Can and Prost Dis 2010 pp 1-7.

66. Dang and Eisenman, MYC and the Pathway to Cancer, CSHL Press, 2014

67. Das et al, Targeting Prostate Cancer Using Bispecific T-Cell Engagers against Prostate-Specific Membrane Antigen, ACS Pharmacol. Transl. Sci., July 2023

68. Das S., et al, MDA-9/syntenin: a positive gatekeeper of melanoma metastasis, Frontiers in Bioscience 17, 1-15, January 1, 2012.

69. Das, P., R. Singal, DNA Methylation and Cancer, Jrl Clin Onco, Nov 2004.

70. Das, S., et al, Therapeutics, Targets, and Chemical Biology Raf Kinase Inhibitor RKIP Inhibits MDA-9/Syntenin-Mediated Metastasis in Melanoma, Cancer Res Published Online First October 11, 2012.

71. Datsi et al, Interleukin-31: The “itchy” cytokine in inflammation and therapy, Allergy. 2021;76:2982–2997

72. De Palma and Lewis, Macrophage Regulation of Tumor Responses to Anticancer Therapies, Cancer Cell, March 18, 2013

73. Deutsch, E., et al, Environmental. Genetic, and Molecular Features of Prostate Cancer, Lancet, 2004, pp 303-313.

74. DeVita, Hellman, and Rosenberg, Cancer: Principles & Practice of Oncology, 9th Ed, Lippincott (New York) 2011.

75. di Bernardo, D., et al, Chemogeneomic Profiling on a Genome Wide Scale Using Reverse Engineered Gene Networks, Nature Biotech, V 23, 2005, pp 377-383.

76. Di Croce, L., et al, Methyltransferase Recruitment and DNA Hypermethylation of Target Promoters by an Oncogenic Transcription Factor, SCIENCE VOL 295 8 FEBRUARY 2002.

77. DiNardo and Ruffel, Macrophages as regulators of tumour immunity and immunotherapy, Nature Reviews Immunology, volume 19, pages369–382 (2019)

78. Djebbari, A., J. Quackenbush, Seeded Bayesian Networks, BioMed Cent Sys Bio 2008 pp 1-13.

79. Doldi et al, Integrated gene and miRNA expression analysis of prostate cancer associated fibroblasts supports a prominent role for interleukin-6 in fibroblast activation, Oncotarget, Vol. 6, No. 31, 2015

80. Donkena K., et al, Oxidative Stress and DNA Methylation in Prostate Cancer, Obs & Gyn Int 2010, pp.

81. Doug, Targeting Tumor-Associated Macrophages (Tams) Reprogramming for Cancer Metastasis Therapy, J Pharm Pharmaceutics 6 2019

82. Easton, J., P. Houghton, mTOR and Cancer Therapy, Oncogene, 2006, pp 6436-6446.

83. Editorial, Precision medicine meets cancer vaccines, Nature Medicine, June 2023

84. Ehrlich, M., DNA methylation in cancer: too much, but also too little, Oncogene (2002) 21, 5400 – 5413.

85. Eisel et al, Cognate Interaction with CD4+ T Cells Instructs Tumor-Associated Macrophages to Acquire M1-Like Phenotype, Frontiers in Immuno, Feb 2019

86. Epelman et al, Origin and Functions of Tissue Macrophages, Immunity, 2014

87. Esteller, M., Epigenetics in Cancer, NEJM, March 2008.

88. Etzerodt et al, Specific targeting of CD163+ TAMs mobilizes inflammatory monocytes and promotes T cell–mediated tumor regression, Jrl Experimental Medicine, August 2019

89. Facciola et al, An Overview of Vaccine Adjuvants: Current Evidence and Future Perspectives, Vaccines 2022

90. Feero, W. G., et al, Genomic Medicine – An Updated Primer, NEJM, 2010, pp 2001-2011.

91. Feinberg, A., et al, The epigenetic progenitor origin of human cancer, Nature Reviews, Genetics Volume 7, January 2006.

92. Fiedler, K., C. Brunner, The role of transcription factors in the guidance of granulopoiesis, Am J Blood Res 2012;2:57-65

93. Finn, O., Cancer Immunology, NEJM V 358, 2008 pp 2704-2715.

94. Fitzgerald and Patrono, The Coxibs, Selective inhibitors of Cyclooxygenases-2, NEJM, Aug 9, 2001

95. Fouad and Aanel, Revisiting the hallmarks of cancer, Am J Cancer Res 2017;7:1016-1036

96. Franken et al, Macrophages: sentinels and regulators of the immune system, Cellular Microbiology, 2016

97. Fridlender and Albelda, Tumor-associated neutrophils: friend or foe?, Carcinogenesis vol.33 no.5 pp.949–955, 2012

98. Frohling, S., Chromosomal Abnormalities in Cancer, NEJM 2008, pp 722-734.

99. Fryczkowski et al, Circulating Levels of Omentin, Leptin, VEGF, and HGF and Their Clinical Relevance with PSA Marker in Prostate Cancer, Disease Markers Volume 2018, Article ID 3852401, 9 pages

100. Fujimura et al, Tumor-Associated Macrophages: Therapeutic Targets for Skin Cancer, Front in Oncology, January 2018 , Volume 8 , Article 3

101. Galluzzi et al, Classification of current anticancer immunotherapies, Oncotarget, Vol. 5, No. 24, 2014

102. Gal-Yam, E., et al, Cancer Epigenetics: Modifications, Screening, and Therapy, Annu. Rev. Med. 2008. 59:267–80.

103. Gebhardt et al, Stem-like exhausted and memory CD8+ T cells in cancer, Nature Reviews Cancer, 2023

104. Ghasemi et al, Cytokine armed dendritic cell progenitors for antigen agnostic cancer immunotherapy, Nature Cancer, 2022

105. Gilboa, The promise of cancer vaccines, Nature Review Cancer, 2004

106. Goldberg et al, G-CSF and Neutrophils Are Nonredundant Mediators of Murine Experimental Autoimmune Uveoretinitis, The American Journal of Pathology, Vol. 186, No. 1, January 2016

107. Goldberg, A., Protein degradation and protection against misfolded or damaged proteins, Nature, Vol 426, 18/25 December 2003.

108. Gonzalgo, M., et al, Prostate Cancer Detection by GSTP1 Methylation Analysis of Post biopsy Urine Specimen, Clin Can Res, 2003, pp 2673-2677.

109. Gottwein, E., B. Cullen, Viral and Cellular MicroRNAs as Determinants of Viral Pathogenesis and Immunity, Cell Host 2008, pp 375-387.

110. Greaves et al, Current understanding of molecular and cellular mechanisms in fibroplasia and angiogenesis during acute wound healing, Derm. Sci, Aug 2013

111. Green, Cell Death, CSHL Press (New York) 2018.

112. Greenberg, P, et al, Revised International Prognostic Scoring System for Myelodysplastic Syndromes, BLOOD, 20 SEPTEMBER 2012 VOLUME 120, NUMBER 12.

113. Gregory and Houghton, Tumor-Associated Neutrophils: New Targets for Cancer Therapy, Cancer Res; 71 April 1, 2011

114. Grivennikov et al, Immunity, Inflammation, and Cancer, Cell 140, 883–899, March 19, 2010

115. Grochow, J., M.. Kellis, Network Motif Discovery Using Subgraph Enumeration and Symmetry Breaking, Speed and Huang Ed, RECOMB Springer 2007 pp 92-106.

116. Guo et al, Metabolic reprogramming of macrophages and its involvement in inflammation diseases, EXCLI Journal 2021

117. Guo et al, Therapeutic Cancer Vaccines: Past, Present and Future, Adv Cancer Res. 2013; 119: 421–475

118. Guo et al, Vaccinia virus-mediated cancer immunotherapy: cancer vaccines and oncolytics, Journal for ImmunoTherapy of Cancer (2019) 7:6

119. Gupta and Massagué, Cancer Metastasis: Building a Framework, Cell 127, November 17, 2006

120. Gupta et al, Recent Advances in Cancer Vaccines: Challenges, Achievements, and Futuristic Prospects, Vaccines, 2022

121. Gupta, R., Adipocytes, Current Biology, V 24, N 20,

122. Hahn, P., M., Novak, Development of Brown and White Adipose Tissue, Jour Lipid Res V 16, 1975, pp 79 91.

123. Han et al, PD-1/PD-L1 pathway: current researches in cancer, Am J Cancer Res 2020;10(3):727-742

124. Hanada et al, Prognostic value of tumor-associated macrophage count in human bladder cancer., Int J Urol. 2000 Jul;7:263-9.

125. Hanahan and Coussens, Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment, Cancer Cell 21, March 20, 2012

126. Hanley, J., B., McNeil, The Meaning and Use of the Area under a Receiver Operating Curve, Radiology 1982 V 143, pp 29-36.

127. Hassler, M., G. Egger, Epigenomics of cancer e emerging new concepts, Biochimie 94 (2012) 2219e2230.

128. Hay, N., The Akt-mTOR tango and its relevance to Cancer, Cancer Cell, V 8, 2005, pp 179-183.

129. Hayden and Ghosh, NF-κB in immunobiology, Cell Research (2011) 21:223-244

130. He, L., G. Hannon, MicroRNAs: Small RNAs with a Big Role in Gene Regulation, Nature, 2004 pp 522-532.

131. Herrero-Cervera et al, Neutrophils in chronic inflammatory diseases, Cellular & Molecular Immunology (2022) 19:177 – 191

132. Heslop, H., et al, Immunotherapy of Hematologic Malignancy, Hematology 2003.

133. Hiam-Galvez et al, Systemic immunity in cancer, Nature Reviews, Cancer Volume 21, June 2021, 345

134. Hillman et al, Hematology in Clinical Practice, 5th Ed, McGraw Hill, 2011

135. Hirayama et al, The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis, Molecular Sciences, 2019

136. Holash et al, VEGF-Trap: A VEGF blocker with potent antitumor effects, PNAS August 20, 2002 vol. 99 no. 17 11393–11398

137. Honda et al, Expression of E2A-HLF Chimeric Protein Induced T-Cell Apoptosis, B-Cell Maturation Arrest, and Development of Acute Lymphoblastic Leukemia, Blood, Vol 93, No 9 (May 1), 1999: pp 2780-2790

138. Hosel and Schmid, The complexity of NF-κB signaling in inflammation and cancer, Molecular Cancer 2013, 12:86

139. Houben, M., et al, Absence of Classical MAP Kinase Pathway Signalling in Merkel Cell Carcinoma, Journal of Investigative Dermatology (2006) 126, 1135–1142.

140. Hsu. P, David M. Sabatini1,2,3 Cancer Cell Metabolism: Warburg and Beyond, Cell 134, September 5, 2008.

141. Huang et al, CCL5 derived from tumor-associated macrophages promotes prostate cancer stem cells and metastasis via activating β-catenin/STAT3 signaling, Cell Death and Disease (2020) 11:234

142. Huang et al, Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy, PNAS , October 23, 2012 , vol. 109 , no. 43

143. Huang, C., et al, A Study of Cancer Related MicroRNAs through Expression Data and Literature Search, World Acad Sci & Engr, 2009 254-256.

144. Huang, S., et al, Blockade of NF-kB Activity in Human Prostate Cancer Cells is Associated with Suppression of Angiogenesis, Invasion, and Metastasis, Oncogene, V 20, 2001, pp 4118-4197.

145. Huff and Zaidi, Vaccine boosts T cells that target pancreatic tumours, Nature | Vol 618 | 1 June 2023

146. Hwangbo, C. et al, mda-9/Syntenin Protein Positively Regulates the Activation of Akt Protein by Facilitating Integrin-linked Kinase Adaptor Function during Adhesion to Type I Collagen, Volume 286 Number 38 Journal Of Biological Chemistry, September 23, 2011.

147. Introna, M., et al, Repeated infusions of donor-derived cytokine-induced killer cells in patients relapsing after allogeneic stem cell transplantation: a phase I study, Haematologica 2007; 92:952-959.

148. Issa, J-P., Epigenetic Changes in the Myelodysplastic Syndrome, Hematol Oncol Clin North Am. Author manuscript; available in PMC 2011 April 1.

149. Iwata, T., et al, Myc Overexpression Induces Prostatic Intraepithelial Neoplasia and Loss of Nkx3.1 in Mouse Luminal Epithelial Cells, Open Access, PLOS,V 5, 2010, pp e9427

150. Jakel, C., et al, Clinical Studies Applying Cytokine-Induced Killer Cells for the Treatment of Renal Cell Carcinoma, Clinical and Developmental Immunology, Volume 2012.

151. Jansen, R., et al, A Bayesian Networks Approach for Predicting Protein-Protein Interactions from Genomic Data, Science, 2003 pp 449-453.

152. Jiang et al, PD-1 and PDL-1 in cancer immunotherapy: clinical implications and future considerations, Human Vaccines & Immunotherapeutics, 2019

153. Jiang, J., et al, Cytokine-induced killer cells promote antitumor immunity, Journal of Translational Medicine 2013, 11:83.

154. Jiang, Y., et al, Aberrant DNA methylation is a dominant mechanism in MDS progression to Aml, Blood, 5 February 2009, Volume 113, Number 6.

155. Jones, P., M. Gonzalgo, Altered DNA methylation and genome instability: A new pathway to cancer?, Proc. Natl. Acad. Sci. USA Vol. 94, pp. 2103–2105, March 1997

156. Jones, P., S. Baylin, The Epigenomics of Cancer, Cell, 2007 pp 683-692.

157. Jordan, C., et al, Cancer Stem Cells, NEJM 2006, pp 1253-1262.

158. Josson et al, Stromal fibroblast-derived miR-409 promotes epithelial-tomesenchymal transition and prostate tumorigenesis, Oncogene (2015) 34, 2690–2699

159. Joyce and Feron, T cell exclusion, immune privilege, and the tumor microenvironment, Science, 3 April 2015 • Vol 348 Issue 6230

160. Junttila1 & de Sauvage, Influence of tumour micro-environment heterogeneity on therapeutic response, Nature, Sept 2013

161. Kaiser, Taking a Shot, Science, April 2022

162. Kalinski, Tumor Immune Microenvironment in Cancer, Springer, 2017

163. Kanamori, Y., et al, Correlation Between Loss of PTEN Expression and Akt Phosphorylation in Endometrial Carcinoma, Clin Can Res, V 7, 2001, pp 892-895.

164. Kang et al, The role of the tumor microenvironment in bladder cancer development and progression, Transl Cancer Res 2017;6 (Suppl 4)

165. Karaman et al, Vascular endothelial growth factor signaling in development and disease, Development (2018) 145

166. Karp et al, Handbook of Targeted Cancer Therapy and Immunotherapy, 2nd Ed, Lippincott, 2018

167. Karp et al, Targeted Cancer Therapy and Immunotherapy, Walters Kluwer, 2023

168. Kelly and O'Neil, Metabolic reprogramming in macrophages and dendritic cells in innate immunity, Cell Research, 2015

169. Kerbel, Tumor Angiogenesis, NEJM, May 2008

170. Kharas et al, Leukemia and Lymphoma, CSHL Press, 2021

171. Kim et al, The Expression of Tumor-Associated Macrophages in Papillary Thyroid Carcinoma, Endocrinol Metab 2013;28:192-198

172. Kim, H., et al, Antitumor activity of cytokine-induced killer cells against human lung cancer, International Immunopharmacology 7 (2007) 1802–1807.

173. Klein and Sato, The HLA System, NEJM, Sept 2000

174. Knezvic, D., et al, Analytical validation of the Oncotype DX prostate cancer assay – a clinical RT-CR assay optimized for prostate needle biopsies, BMC Genomics 2013, 14:690.

175. Kohler et al, G-CSF–mediated thrombopoietin release triggers neutrophil motility and mobilization from bone marrow via induction of Cxcr2 ligands, Blood, 21 April 2011 Volume 117, Number 16

176. Koreth J., et al, Role of Reduced-Intensity Conditioning Allogeneic Hematopoietic Stem-Cell Transplantation in Older Patients With De Novo Myelodysplastic Syndromes: An International Collaborative Decision Analysis, JOURNAL OF CLINICAL ONCOLOGY, June 2013.

177. Kovaleva et al, Tumor Associated Macrophages in Kidney Cancer, Analytical Cellular Pathology, Volume 2016, Article ID 9307549

178. Krause, D., R. VanEtten, Tyrosine Kinases as Targets for Cancer Therapy, NEJM 2005 pp 172-187.

179. Kundu and Surh, Inflammation: Gearing the journey to cancer, Mutation Research 659 (2008) 15–30

180. Laan et al, role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-a, Eur Respir J 2003; 21: 387–393

181. Labanieh and Mackall, CAR immune cells: design principles, resistance and the next generation, Nature, Feb 2023

182. Lacovara J., et al, Fibronectin Enhancement of Directed Migration of B16 Melanoma Cells, Cancer Research, 1984.

183. Laird, P., R. Jaenisch, DNA Methylation and Cancer, Human Molecular Genetics, Vol 3, 1487-95, 1999.

184. Lam, E., et al, FOXO Transcription Factors, Biochem Soc Trans 2006, pp 722-726.

185. Lamprecht et al, Teaming Up for Trouble: Cancer Cells, Transforming Growth Factor-β1 Signaling and the Epigenetic Corruption of Stromal Naïve Fibroblasts, Cancers 2018, 10, 61

186. Landskron et al, Chronic Inflammation and Cytokines in the Tumor Microenvironment, Jrl Immuno Research, 2014

187. Larionova et al, Interaction of tumor-associated macrophages and cancer chemotherapy, Oncoimmunology, 2019, VOL. 8, NO. 7,

188. Lau et al, Cancer Stem Cells and Their Microenvironment: Biology and Therapeutic Implications, Stem Cells International, Volume 2017, Article ID 3714190, 11 pages

189. Laviron and Boissonnas, Ontogeny of Tumor-Associated Macrophages, Frton in Immun, July 2019

190. Lazarov et al, Physiology and diseases of tissue-resident macrophages, Nature June 2023

191. Lecis et al, Immune Checkpoint Ligand Reverse Signaling: Looking Back to Go Forward in Cancer Therapy, Cancers 2019,11, 624

192. Lee et al, The Expression of Tumor-Associated Macrophages in Papillary Thyroid Carcinoma, Endocrinol Metab 2013;28:192-198

193. Leko and Rosenberg, Identifying and Targeting Human Tumor Antigens for T Cell Based Immunotherapy of Solid Tumors, Cancer Cell. 2020 October 12

194. Leonard and Schreiber, Cytokines, CSHL, 2018

195. Levine, A., A. Puzio-Kuter, The Control of the Metabolic Switch in Cancers by Oncogenes and Tumor Suppressor Genes, Science, 2010, pp 1340-1344.

196. Li et al, Adipocytes promote tumor progression and induce PD-L1 expression via TNF-α/IL-6 signaling, Cancer Cell Int (2020) 20:179

197. Li et al, G-CSF as a potential early biomarker for diagnosis of bloodstream infection, J. Clin. Lab. Anal. 2021;35:e23592.

198. Li et al, The dynamic role of platelets in cancer progression and their therapeutic implications, Nature Reviews Cancer, 2023

199. Li, Q., Selective anticancer strategies via intervention of the death pathways relevant to cell transformation, Cell Death and Differentiation (2008) 15, 1197–1210.

200. Lin et al, Cancer vaccines: the next immunotherapy frontier, Nature Cancer, Aug 2022

201. Lin et al, Macrophages Regulate the Angiogenic Switch in a Mouse Model of Breast Cancer, Cancer Res 2006; 66: . December 1, 2006

202. Linehan and Ricketts, The Metabolic Basis of Kidney Cancer, Semin Cancer Biol. 2013 February; 23: 46-55.

203. Linehan et al, The genetic basis of kidney cancer: a metabolic disease, Nat Rev Urol. 2010 May; 7: 277–285

204. Linn, Y., K. Hui, Cytokine-Induced NK-Like T Cells: From Bench to Bedside, Journal of Biomedicine and Biotechnology, Volume 2010.

205. List, A., et al, Efficacy of Lenalidomide in Myelodysplastic Syndromes, NEJM, Feb 2005.

206. Liu et al, Cancer vaccines as promising immuno-therapeutics: platforms and current progress, Journal of Hematology & Oncology (2022) 15:28

207. Liu et al, Cancer vaccines: the next immunotherapy frontier, Nature Cancer, VOL 3, August 2022, 911–926

208. Liu et al, Cancer-Associated Fibroblasts Build and Secure the Tumor Microenvironment, Frontiers in Cell and Developmental Biology, 1 April 2019, Volume 7, Article 60

209. Liu et al, G-CSF promotes the viability and angiogenesis of injured liver via direct effects on the liver cells,

210. Liu et al, Interleukin-6 and the Granulocyte Colony-Stimulating Factor Receptor Are Major Independent Regulators of Granulopoiesis In Vivo But Are Not Required for Lineage Commitment or Terminal Differentiation, Blood, Vol 90, No 7 (October 1), 1997: pp 2583-2590

211. Liu et al, Metabolic reprogramming in macrophage responses, Biomarker Research (2021) 9:1

212. Liu et al, mRNA-based cancer therapeutics, Nature Reviews Cancer , Volume 23 , August 2023 , 526–543

213. Liu et al, Novel strategies for cancer immunotherapy: counter-immunoediting therapy, Journal of Hematology & Oncology (2023) 16:38

214. Liu, L., et al, Randomized Study of Autologous Cytokine-Induced Killer Cell Immunotherapy in Metastatic Renal Carcinoma, Clin Cancer Res Published OnlineFirst January 24, 2012.

215. Liu, W., et al, The Melanoma-Associated Antigen A3 Mediates Fibronectin-Controlled Cancer Progression and Metastasis, Cancer Res 2008;68:8104-8112. Published online September 30, 2008.

216. Lobo, N., et al, The Biology of Cancer Stem Cells, Ann Rev Cell Dev 2007 pp 675-699.

217. Look, A., A leukemogenic twist for GATA1, Nature Genetics 32, 83 - 84 (2002)

218. LoPiccolo, J., et al, Targeting the PI3K/Akt/mTOR Pathway, Drug Res Up, 2008, pp 32-50.

219. Lorentzen et al, Clinical advances and ongoing trials of mRNA vaccines for cancer treatment, Lancet Oncology October 2022

220. Lu, Y., et al, A Bayesian Approach to Simultaneously Adjusting for Verification, Statst Med 2010 V 29, pp 2532-2543.

221. Lubbert, M., et al, Low-Dose Decitabine Versus Best Supportive Care in Elderly Patients With Intermediate- or High-Risk Myelodysplastic Syndrome (MDS) Ineligible for Intensive Chemotherapy: Final Results of the Randomized Phase III Study of the European Organisation for Research and Treatment of Cancer Leukemia Group and the German MDS Study Group, J Clin Oncol 29:1987-1996. 2011.

222. Luca, M., et al, Expression of Interleukin-8 by Human Melanoma Cells Up-Regulates MMP-2 Activity and Increases Tumor Growth and Metastasis, American Journal of Pathology, Vol. 151, No. 4, October 1997.

223. Luczak, M., P. Jagodzinski, The role of DNA methylation in cancer development, Folia Histochemica Et Cytobiologica Vol. 44, No. 3, 2006 pp. 143-154.

224. Maddaluno, Fibroblast growth factors: key players in regeneration and tissue repair, Development (2017) 144, 4047-4060

225. Madden et al, Taking the Myc out of cancer: toward therapeutic strategies to directly inhibit c-Myc, Molecular Cancer (2021) 20:3

226. Maira, S., et al, PI3K Inhibitors for Cancer Treatment, Biochem Soc Trans, V 37, 2009, pp 265-272.

227. Mariuzza et al, The structural basis of T-cell receptor (TCR) activation: An enduring enigma, JBC Reviews, 2020

228. Marten, A., Enhanced lytic activity of cytokineinduced killer cells against multiple myeloma cells after co-culture with idiotype-pulsed dendritic cells, Haematologica 2001; 86:1029-1037.

229. Martinez and Gordon, The M1 and M2 paradigm of macrophage activation: time for reassessment, F1000Prime Reports 2014, 6:13

230. Martinez-Vincente, M., et al, Protein degradation and aging, Experimental Gerontology 40 (2005) 622–633.

231. Masucci et al, Tumor Associated Neutrophils. Their Role in Tumorigenesis, Metastasis, Prognosis and Therapy, Frontiers in Oncology, 15 Nov 2019

232. Matsukawa and Yoshinaga, Neutrophils as a source of cytokines in inflammation, Histol Histopathol (1999) 14: 511-516

233. Maxson and Tyner, Genomics of chronic neutrophilic leukemia, Blood, 9 February 2017, Volume 129, Number 6

234. Mazar, J., et al, Epigenetic Regulation of MicroRNA Genes and the Role of miR-34b in Cell Invasion and Motility in Human Melanoma, PLOS One, September 2011, Volume 6, Issue 9.

235. McCabe, M., et al, Cancer DNA Methylation: Molecular Mechanisms and Clinical Implications, Clinical Cancer Res 3927 2009;15 June 15, 2009.

236. McCauley et al, Macrophage subtype and cytokine expression characterization during the acute inflammatory phase of mouse bone fracture repair, J Orthop Res. 2020;38:1693–1702

237. McGillis, J., White Adipose Tissue, Inert No More, Endocrin, V 146 2005 pp. 2154 - 2156.

238. McIntosh, M, N., Urban, A Parametric Empirical Bayes Method for Cancer Screening, Biostat 2003 V 4, pp 27-40.

239. McMenamin, M., et al, Loss of PTEN Expression in Paraffin-embedded Primary Prostate Cancer, Can Res, V 59, 1999, pp 4291-4296.

240. Medzhitov, Innate Immunity and Inflammation, CSHL Press, 2015

241. Melief et al, Therapeutic cancer vaccines, J Clin Invest. 2015;125:3401-3412

242. Mellgard et al, Lessons from withdrawn accelerated approvals in oncology, Nature Cancer, 2024

243. Mello, C., Return of the RNAi World: Rethinking Gene Expression and Evolution, Nobel Laureate Lecture 2006.

244. Mempei et al, How chemokines organize the tumor microenvironment, Nature Reviews Cancer, 2023

245. Mendelsohn and Baselga, The EGF receptor family as targets for cancer therapy, Oncogene (2000) 19, 6550 – 6565

246. Mendelsohn and Baselga, The EGF receptor family as targets for cancer therapy, Oncogene (2000) 19, 6550 – 6565

247. Mendelsohn, et al, Molecular Basis of Cancer, Saunders (New York) 2015.

248. Mercogliano et al, Emerging Targeted Therapies for HER2-Positive Breast Cancer, Cancers 2023

249. Metcalf, On Hematopoietic Stem Cell Fate, Immunity 26, June 2007

250. Metcalfe, Mast cells and mastocytosis, Blood, 2008 112: 946-956

251. Metzker, M., Sequencing Technologies, Nature Rev V 11, 2010, pp 31-46.

252. Metzner et al, Fibroblast Growth Factor Receptors as Therapeutic Targets in Human Melanoma: Synergism with BRAF Inhibition, Journal of Investigative Dermatology (2011), Volume 131

253. Milo et al, Cell Biology by the Numbers, Garland, 2016

254. Milo, R., et al, Network Motifs: Simple Building Blocks of Complex Networks, Science, 2002, pp 824-827.

255. Mizuno, S., et al, Expression of DNA methyltransferases DNMT1,3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia, BLOOD, 1 MARCH 2001 z VOLUME 97, NUMBER 5.

256. Mjolsness, E., et al Gene Regulation Networks with Signaling and Cell Division in Development Satiations, IEEE Biomed Engr Houston TX Oct 2002.

257. Molina-Cerrillo et al, Tumor-Associated Macrophages: "Good Cop-Bad Cop", Cancer · February 2019

258. Monteran and Erez, The Dark Side of Fibroblasts: Cancer-Associated Fibroblasts as Mediators of Immunosuppression in the Tumor Microenvironment, Frontiers in Immunology, August 2019 | Volume 10 | Article 1835

259. Montparler, R., Cancer epigenetics, Oncogene (2003) 22, 6479–6483.

260. Mooi, W., D. Peeper, Oncogene Induced Cell Senescence, NEJM, 2006 pp 1037-1045.

261. Mora et al, Strategies to Interfere with Tumor Metabolism through the Interplay of Innate and Adaptive Immunity, Cells 2019, 8, 445

262. Morceau, T., et al, Pro-Inflammatory Cytokine-Mediated Anemia: Regarding Molecular Mechanisms of Erythropoiesis, Mediators of Inflammation, Volume 2009, Article ID 405016

263. Morgan, The Cell Cycle, Sinauer, 2007

264. Mosquera, J., et al, Prevalence of TMPRSS2-ERG Fusion Prostate Cancer among Men Undergoing Prostate Biopsy in the United States, Clin Can Res, V 15, 2009, 4706-4711.

265. Mowers et al, Autophagy in cancer metastasis, Oncogene (2017) 36, 1619–1630

266. Murekatete et al, Targeting Insulin-Like Growth Factor-I and Extracellular Matrix Interactions in Melanoma Progression, Scientific Reports, 2018

267. Murgaski et al, Unleashing Tumour-Dendritic Cells to Fight Cancer by Tackling Their Three A's: Abundance, Activation and Antigen-Delivery, Cancers 2019, 11, 670

268. Murrell, J., Understanding Rate of Chemical Reactions, University of Sussex.

269. Murtaza M et al, Noninvasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA (2013) Nature.

270. Nelson, E., et al, Inhibition of Akt Pathways in the Treatment of Prostate Cancer, 2007, Pros Can and Pros Dis, pp 331-339.

271. Ngambenjawong et al, Progress in tumor-associated macrophage (TAM)-targeted therapeutics, Adv Drug Deliv Rev. 2017 May 15; 114: 206–221.

272. Niimi et al, Hyperactivation of the RAS signaling pathway in myelodysplastic syndrome with AML1/RUNX1 point mutations, Leukemia (2006) 20, 635–644

273. Noh et al, Crosstalk Between Prostate Cancer Cells and TumorAssociated Fibroblasts Enhances the Malignancy by Inhibiting the Tumor Suppressor PLZF, Cancers 2020, 12, 1083

274. Noy and Pollard, Tumor-Associated Macrophages: From Mechanisms to Therapy, Immunity 41, July 17, 2014

275. Nusse, R., Relays at the Membrane, Nature, 2005, V 438, pp 747-748.

276. Nyberg et al, Tumor microenvironment and angiogenesis, Frontiers in Bioscience 13, 6537-6553, May 1, 2008

277. O'Shaughnessy, J, et al, Treatment and Prevention of Intraepithelial Neoplasia: An Important Target for Accelerated New Agent Development, Clinical Cancer Research, Vol. 8, 314–346, February 2002.

278. Ogoshi, K., et al, Genome-wide profiling of DNA methylation in human cancer cells, Genomics (2011).

279. Olnes, M., E. Sloand, Targeting Immune Dysregulation in Myelodysplastic Syndromes, JAMA, Feb 2011.

280. Olsen et al, Fibroblast Growth Factor (FGF) Homologous Factors Share Structural but Not Functional Homology with FGFs, The Journal Of Biological Chemistry Vol. 278, No. 36, Issue of September 5, pp. 34226–34236, 2003

281. Ornitz and Ito, The Fibroblast Growth Factor signaling pathway, WIREs Dev Biol 2015, 4:215–266

282. Ornitz and Itoh, Fibroblast growth factors, Genome Biology 2001

283. Ozkumur, E., Inertial Focusing for Tumor Antigen–Dependent and –Independent Sorting of Rare Circulating Tumor Cells, Sci Transl Med 3 April 2013: Vol. 5, Issue 179, p. 179.

284. Pabst, T., B. Mueller, Complexity of CEBPA Dysregulation in Human Acute Myeloid Leukemia, Clin Cancer Res 2009;15 September 1, 2009

285. Page et al, Immune Modulation in Cancer with Antibodies, Immune Modulation in Cancer, https://www.annualreviews.org/ October 2013

286. Pakasama, P, et al, Ex vivo generation of cytokine-induced killer cells (CD3+ CD56+) from post-stem cell transplant pediatric patients against autologous–Epstein–Barr virus–transformed lymphoblastoid cell lines, Pediatr Transplantation 2007: 11: 511–517.

287. Pali, S., K. Robertson, Epigenetic Control of Tumor Suppression, Critical Reviews™ in Eukaryotic Gene Expression, 17:295–316 (2007).

288. Pallangyo et al, IKKβ acts as a tumor suppressor in cancer-associated fibroblasts during intestinal tumorigenesis, J. Exp. Med. 2015 Vol. 212 No. 13 2253–2266

289. Pallerla et al, Cancer Vaccines, Treatment of the Future: With Emphasis on HER2-Positive Breast Cancer, Int. J. Mol. Sci. 2021, 22, 779

290. Palma et al, Gene Regulatory Network Modeling of Macrophage Differentiation Corroborates the Continuum Hypothesis of Polarization States, Frontiers in Physiology, 2018

291. Pang et al, Hematopoietic stem cell and progenitor cell mechanisms in myelodysplastic syndromes, PNAS | February 19, 2013 | vol. 110 | no. 8 | 3011–3016.

292. Papatsoris, A., et al, The Power and Promise of "Rewiring" the Mitogen Activated Protein Kinase Network in Prostate Cancer Theraputics, Mol Can Ther, V 6, 2007, pp 811-819.

293. Paston et al, Cancer vaccines, adjuvants and delivery systems, Frontiers in Immunology, | March 2021

294. Patel et al, Neuroendocrine Differentiation of Prostate Cancer, An Intriguing Example of Tumor Evolution at Play, Cancers 2019, 11, 1405

295. Pathria et al, Targeting Tumor-Associated Macrophages in Cancer, Trends in Immunology, March 2019

296. Payne, S., C. Kemp, Tumor Suppressor Genetics, Carcinogenesis, V 26, 2005, pp 2031-2045.

297. Pecorino, L., Molecular Biology of Cancer, Oxford (New York) 2008.

298. Pelengaris et al, c-MYC: more than just a matter of life and death, Nat Rev Cancer 2, 764–776 (2002).

299. Peruzzi B, Bottaro DP. Targeting the c-Met signaling pathway in cancer. Clin Cancer Res 2006 Jun 15;12:3657–60

300. Peter W. Laird, P., R. Jaenisch, DNA Methylation and Cancer, Human Molecular Genetics, Vol 3, 1487-95, 1994. http://hmg.oxfordjournals.org/content/3/suppl_1/1487.abstract?sid=0ef56eee-6b89-4808-9ded-2004a353783f

301. Pezzella et al, Cancer Biology, Oxford, 2019

302. Pietras et al, PDGF receptors as cancer drug targets, CANCER CELL : MAY 2003 • VOL. 3

303. Pievani, A. et al, Dual functional capability of CD3+CD56+ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity, Blood, Prepublished online August 5, 2011.

304. Planet, P. et al, Systematic Analysis of DNA Microarray Data: Ordering and Interpreting Patterns of Gene Expression, Cold Spring Harbor, Genome Research, 2001 pp 1149-1155.

305. Poh and Ernst, Targeting Macrophages in Cancer: From Bench to Bedside, Front in Oncology, 12 March 2018

306. Poliseno, L., et al, Identification of the miR-106b MicroRNA Cluster as a Proto Oncogenic PTEN Targeting Intron That Cooperates with the Host Gene MCM7 in Transformation, Sci Sig 2010, pp 29- .

307. Prendergast and Jaffee, Cancer Immunotherapy, Academic, 2013

308. Prystaz et al, Distinct Effects of IL-6 Classic and Trans-Signaling in Bone Fracture Healing, The American Journal of Pathology, Vol. 188, No. 2, February 2018

309. Purushoth et al, Cancer Progression Related with Tumor-associated Macrophages, Cancer and Oncology Research 4: 53-63, 2016

310. Putzi, M., A. De Marzo, Morphologic Transitions Between Proliferative Inflammatory Atrophy and High Grade Prostatic Intraepithelial Neoplasia, Adult Uro 2000 pp 828-832.

311. Quaranta and Schmid, Macrophage-Mediated Subversion of Anti-Tumour Immunity, Cells 2019, 8, 747

312. Radu, A., et al, PTEN Induces Cell Cycle Arrest by Decreasing the Level and Nuclear Localization of Cyclin D1, Mol and Cell Bio, V 23, 203, pp 6139-6149.

313. Rahim et al, Molecular Regulation of Bone Marrow Metastasis in Prostate and Breast Cancer, Bone Marrow Research, Volume 2014, Article ID 405920, 12 pages

314. Rajala, M., P. Scherer, Minireview: The Adipocyte, Endocrin, V 144, pp 3765 3773.

315. Ramos, D., et al, Analysis of Integrin Receptors for Laminin and Type IV Collagen on MetastaticB16 Melanoma Cells, Cancer Research, 1990.

316. Rastogi et al, Vaccines as treatments for prostate cancer, Nature Reviews Urology, September 2023

317. Rettinger, E., et al, Cytotoxic capacity of IL-15-stimulated cytokine-induced killer cells against human acute myeloid leukemia and rhabdomyosarcoma in humanized preclinical mouse models, Front Oncology April 2012.

318. Reya, T., et al, Stem Cells, Cancer, and Cancer Stem Cells, Nature, 2001 pp 105-111.

319. Riabov, et al, Role of tumor associated macrophages in tumorangiogenesis and lymphangiogenesis, Front in Physiology, March 2014

320. Richter et al, Take my breath away—mitochondrial dysfunction drives CD8+ T cell exhaustion, Genes & Immunity, 2024

321. Robertson, K., A. Wolffe, DNA Methylation in Health and Disease, Nat Rev Gen 2000 pp 11-19.

322. Robertson, K., DNA methylation, methyltransferases, and cancer, Oncogene (2001) 20, 3139-3155.

323. Rojas et al, Personalized RNA neoantigen vaccines stimulate T cells in pancreatic cancer, Nature | Vol 618 | 1 June 2023

324. Roma-Rodrigues et al, Targeting Tumor Microenvironment for Cancer Therapy, Int. J. Mol. Sci. 2019, 20, 840

325. Rosales, Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types?, Frontiers in Physiology, 1 February 2018 , Volume 9 , Article 113

326. Rosen, J., C. Jordan, The Increasing Complexity of the Cancer Stem Cell Paradigm, Science, 2009, pp 1670-1672.

327. Rosenberg, S., Gene Therapy for Cancer, JAMA 1992 pp 2416-2419.

328. Rosenberg, S., The Transformed Cell, Putnam (New York) 1992.

329. Roy, S., et al, Inhibition of PI3K/AKT and MAPK/ERK pathways causes Activation of FOXO Transcription Factor, Jrl Mol Sig 2010, pp 1-13.

330. Rubin, M., A. Chinnaiyan, Bioinformatics Approach Leads to the Discovery of the TMPRSS2:ETS gene Fusion in Prostate Cancer, Lab Inv 2006, pp. 1099-1102.

331. Rucci and Angelucci, Prostate Cancer and Bone: The Elective Affinities, BioMed Research International, Volume 2014, Article ID 167035, 14 pages

332. Ruffell1 and Coussens, Macrophages and Therapeutic Resistance in Cancer, Cancer Cell, April 2015

333. Ryder et al, Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer, Endocrine-Related Cancer (2008) 15 1069–1074

334. Sahai et al, A framework for advancing our understanding of cancer-associated fibroblasts, Nature Reviews, Cancer, March 2020, Volume 20

335. Salinero and Rafii, Endothelial cell adaptation in regeneration, Science, Dec 2018

336. Salmi et al, The number and localization of CD68+ and CD163+ macrophages in different stages of cutaneous melanoma, Melanoma Research 2018, Vol 00 No 00

337. Sanford-Crane et al, Fibroblasts as Modulators of Local and Systemic Cancer Metabolism, Cancers 2019, 11, 619

338. Sangiolo, D., Cytokine Induced Killer Cells as Promising Immunotherapy for Solid Tumors, Journal of Cancer 2011, 2.

339. Santos and Butterfield, Dendritic cell-based cancer vaccines, Jrl Immuno, 2018

340. Sarkar, D., et al, mda-9/Syntenin: More than Just a Simple Adapter Protein When It Comes to Cancer Metastasis, Cancer Res 2008; 68: . May 1, 2008.

341. Sasaki et al, Thymoproteasomes produce unique peptide motifs for positive selection of CD8+ T cells, Nature Communications, June 2015

342. Saunthararajah, Y., et al, Evaluation of noncytotoxic DNMT1-depleting therapy in patients with myelodysplastic syndromes, J Clin Invest. doi:10.1172/JCI78789, 2015.

343. Saxena et al, Therapeutic Cancer Vaccines, Nature June 2021

344. Schnell. S, T. Turner, Reaction Kinetics in Intracellular Environments with Macromolecular Crowding, Biophys and Molec Bio vol 85 2004 pp. 235-260.

345. Sengupta et al, Regulation of the mTOR Complex 1 Pathway by Nutrients, Growth Factors, and Stress, Molecular Cell 40, October 22, 2010

346. Seoane, J., et al, Integration of Smad and Forkhead Pathways in the Control of Neuroepithelial and Glioblastoma Cell Proliferation, Cell 2004, pp. 211-223.

347. Setlur, S., et al, Integrative Microarray Analysis of Pathways Dysregulated in Metastatic Prostate Cancer, Cancer Res, V 67, 2007, pp 10296-10303.

348. Sevigani, C., et al, Mammalian microRNAs: A Small World for Fine-tuning Gene Expression, Mam Gene V 17 2006, pp 189-201.

349. Sevli, S., et al, The Function of microRNAs, small but potent molecules, in Human Prostate Cancer, Pros Can and Pros Dis, V 13, 2010, pp 208-217.

350. Sharifi, N., et al, Androgen Deprivation Therapy for Prostate Cancer, JAMA, V 294, 2005, pp 238-244.

351. Sharma, Osteoarthritis of the knee, NEJM, Jan 2021

352. Shen and Rubin, Prostate Cancer, CSHL, 2019

353. Sherbet, Growth Factors and their Receptors in Cell Differentiation, Cancer and Cancer Therapy, Elsevier, 2011

354. Shyam, E. et al, The ERG Gene: A Human Gene Related to the ETS Oncogene, PNAS 1987, pp 6131-6135.

355. Sica, G., Bias in Research Studies, Radiology, 2006 V 238 pp 780-789.

356. Silva et al, Increased peripheral blood neutrophil activation phenotypes and NETosis in critically ill COVID-19 patients, Preprint · January 2021, DOI: 10.1101/2021.01.14.21249831

357. Silvestri et al, A Perspective of Immunotherapy for Prostate Cancer, Cancers 2016

358. Singh et al, EGF receptor ligands: recent advances, F1000 Research 2016.

359. Slingerland, J., M. Pagano, Regulation of Cdk Inhibitor p27 and Its Deregulation in Cancer, Jrl Cell Phys 2000 pp 10-17.

360. Solinas et al, Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation, Volume 86, November 2009 Journal of Leukocyte Biology

361. Sonnet et al, Early aberrant DNA methylation events in a mouse model of acute myeloid leukemia, Genome Medicine 2014, 6:34

362. Spravchikov et al, Glucose Effects on Skin Keratinocytes, Diabetes, Vol. 50, July 2001

363. Steidl et al, Tumor-Associated Macrophages and Survival in Classic Hodgkin's Lymphoma NEJM, March 10, 2010

364. Stein, G., et al, The Molecular Basis of Cell Cycle and Growth Control, Wiley (New York) 1999.

365. Strathdee, G., R., Brown, Aberrant DNA Methylation in Cancer; Potential Clinical Interventions, Exp Rev Mol Med, 2002, pp 1-17.

366. Stressman C., et al, Azacytidine causes complex DNA methylation responses in myeloid leukemia, Mol Cancer Ther. 2008 Sep;7:2998-3005.

367. Studier, J., K. Kappler, A Note on the Neighbor Joining Algorithm, Molecular Biological Evolution, Vol 5 1988 pp 729-731.

368. Styerberg, E., et al, Prediction of Indolent Prostate Cancer, Jrl Uro, 2007, V 177, pp 107-112.

369. Su, L., Early Diagnosis and Treatment of Cancer: Prostate Cancer, Saunders (New York) 2010.

370. Suarez-Carmona et al, EMT and inflammation: inseparable actors of cancer progression, Molecular Oncology 11 (2017) 805–823

371. Sun et al, Evolution of CD8+ T cell receptors (TCR) engineered therapies for treatment of cancer, Cells, 2021

372. Sun et al, The PI3K/AKT/mTOR signaling pathway in osteoarthritis: a narrative review, Osteoarthritis and Cartilage 28 (2020)

373. Suzuki, H. et al, Roles and causes of abnormal DNA methylation in gastrointestinal cancers, Asian Pac J Cancer Prev. 2006 Apr-Jun;7:177-85.

374. Swanson, K, et al, Prostate Specific Antigen, Am Jrl Clin Path, 2006, V 125 pp 331-333.

375. Swiatoniowski, G et al, E-cadherin and Fibronectin Expressions Have No Prognostic Role in Stage II Ductal Breast Cancer, Anticancer Research, 25: 2879-2884 (2005).

376. Szallasi, Z. System Modeling in Cellular Biology: From Concepts to Nuts and Bolts. MIT Press (Cambridge) 2006.

377. Taberlay, P., P. Jones, DNA Methylation and Cancer, Epigenetics and Disease, Progress in Drug Research 67, 2011.

378. Taby, R., J-P Issa, Cancer Epigenetics, CA 2010 pp376-392.

379. Taichman et al, The evolving biology and treatment of prostate cancer, The Journal of Clinical Investigation http://www.jci.org Volume 117 Number 9 September 2007

380. Taichman, Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche, Blood, 1 April 2005, Volume 105, Number 7

381. Takahashi et al, Cancer-associated fibroblasts promote an immunosuppressive microenvironment through the induction and accumulation of protumoral macrophages, Oncotarget, 2017, Vol. 8, (No. 5), pp: 8633-8647, 2016

382. Takayama et al., Increased infiltration of tumor associated macrophages is associated with poor prognosis of bladder carcinoma in situ after intravesical bacillus Calmette-Guerin instillation., J Urol. 2009 Apr;181:1894-900

383. Takeuchi and Ito, Receptor Tyrosine Kinases and Targeted Cancer Therapeutics, Biol. Pharm. Bull. 34 1774—1780 (2011)

384. Tang, D., et al, Prostate Cancer Stem/Progenitor Cells, Mol Car 2007 pp. 1-14.

385. Tay et al, Cellular players of hematopoietic stem cell mobilization in the bone marrow niche, Int J Hematol (2017) 105:129–140

386. Taylor, B., Integrative Genomic Profiling of Human Prostate Cancer, Cancer Cell, V 18, 2010, pp 1-12.

387. Teishima et al, Fibroblast Growth Factor Family in the Progression of Prostate Cancer, J. Clin. Med. 2019, 8, 183

388. Tukan et al, Analysis of Circulating Tumor Cells in Patients with Non-Metastatic High-Risk Prostate Cancer before and after Radiotherapy Using Three Different Enumeration Assays, Cancers 2019 V 11 N 802

389. van den Bosch et al, Alarmin S100A9 Induces Proinflammatory and Catabolic Effects Predominantly in the M1 Macrophages of Human Osteoarthritic Synovium, The Journal of Rheumatology 2016; 43:10

390. van den Bosch, Osteoarthritis year in review 2020: biology, Osteoarthritis and Cartilage 29 (2021) 143e150

391. van der Burg et al, Vaccines for established cancer: overcoming the challenges posed by immune evasion, Nature Reviews Cancer, April 2016

392. van der Leun et al, CD8+ T cell states in human cancer: insights from a single cell analysis, Nature Reviews Cancer, April 2020

393. Varricchi et al, Are Mast Cells MASTers in Cancer?, Front. in Immuno, 2 April 2017

394. Vasiljevic, N , et al, Association between DNA methylation of HSPB1 and death in low Gleason score prostate cancer, Prostate Cancer and Prostatic Disease (2013) 16, 35–40.

395. Vazquez, F., et al, The PTEN Tumor Suppressor Protein, Biochimica et Biophysica, 2000 pp 21-25.

396. Vely, F., E., Vivier, Natural Killer Cell Receptor Signaling Pathway, Sci. STKE 2005 ,

397. Venere et al, Growth Factor Receptors Define Cancer Hierarchies, Cancer Cell 23, February 11, 2013

398. Vicini FA, Abner A, Baglan KL, Kestin LL, Martinez AA. Defining a dose-response relationship with radiotherapy for prostate cancer: is more really better? Int J Radiat Oncol Biol Phys. 2001 Dec 1;51:1200-8.

399. Virtanen, K., et al, Functional Brown Adipose Tissue in Health Adults, NEJM 360 2009 PP 1518 1525.

400. Vis, A., F. Schroder, Key Targets of hormonal treatment of prostate cancer; Part 2 the androgen receptor and 5α reductase, BJUI 2009 pp 1191-1197.

401. Vliestra, R., et al, Frequent Inactivation of PTEN in Prostate Cancer Cell Lines and Xenografts, Can Res, V 58, 1998, pp 2720-2723.

402. Vogelstein, B., K., Kinzler, Cancer Genes and the Pathways They Control, Nature Med, V 10, 2004, pp 789-799.

403. von Vietinghoff and Ley, Homeostatic regulation of blood neutrophil counts, Homeostatic regulation of blood neutrophil counts

404. Wagner, Fibroblasts for all Seasons, Nature, Vol 530, 4 Feb 2016

405. Walter, et al, Clonal Architecture of Secondary Acute Myeloid Leukemia, NEJM, March 2012.

406. Wang et al, Cancer-associated fibroblast regulation of tumor neo-angiogenesis as a therapeutic target in cancer, Oncology Letters 17: 3055-3065, 2019

407. Wang, X, et al, Notch-1 Expressing Cells are Indispensable for Prostatic Branching Morphogenesis, Jrl Bio Chem, V 279, 2004, pp 24733-24744.

408. Wantanabe et al, The role of macrophages in the resolution of inflammation, Jrl Clin Inv July 2019

409. Warboys and Davis, Do inhibitory receptors need to be proximal to stimulatory receptors to function?, Genes & Immunity, 2024

410. Warburg, O., On the Origin of Cancer Cells, Science 24 February 1956, Volume 123, Number 3191.

411. Ward et al, Projected U.S. State-Level Prevalence of Adult Obesity and Severe Obesity, NEJM, 19 Dec 2019

412. Waters et al, Hepatic leukemia factor promotes resistance to cell death: Implications for therapeutics and chronotherapy, Toxicology and Applied Pharmacology, Volume 268, Issue 2, 15 April 2013, Pages 141–148

413. Watson, J, F. Crick, Molecular Structure of Nucleic Acids, Nature, pp 737-738, April 25, 1953.

414. Weinberg, R., The Biology of Cancer, 2nd Ed, Garland (New York) 2013.

415. Werhli, A., D. Husmeier, Reconstructing Gene Regulatory Networks with Bayesian Networks, 2007 Bioinfo and Stat pp 1-45.

416. Wesche et al, Fibroblast growth factors and their receptors in cancer, Biochem. J. (2011) 437, 199–213

417. Whiteside, The tumor microenvironment and its role in promoting tumor growth, Oncogene (2008) 27, 5904–5912

418. Wiegertjes et al, A roadmap to target interleukin-6 in osteoarthritis, Rheumatology 2020;59:2681–2694

419. Witsch et al, Roles for Growth Factors in Cancer Progression, PHYSIOLOGY 25: 85–101, 2010

420. Wolf, D., et al, Transcriptional Down Regulation of c-myc in Human Prostate Carcinoma Cells by the Synthetic Mibolerone, Br Jrl Cancer, V 65, 1992, pp 376-382.

421. Wu et al, The role of macrophages in osteoarthritis and cartilage repair, Osteoarthritis and Cartilage 28 (2020) 544e554

422. Wu et al, Tumor-Associated Neutrophils in Cancer: Going Pro, Tumor-Associated Neutrophils in Cancer: Going Pro

423. Wu., F.X. et al, Modeling Gene Expression From Microarray Expression Data with State Space Equations.

424. Xia, H., Great Potential of MicroRNA in Cancer Stem Cell, Jrl Can Mol 2008 pp 79-89.

425. Xu, Regulation of Inflammatory Signalling in Health and Disease, Springer, 2017

426. Yan et al, IL-6 Cooperates with G-CSF To Induce Protumor Function of Neutrophils in Bone Marrow by Enhancing STAT3 Activation, The Journal of Immunology, 2013

427. Yang et al, Growth Factors and their receptors in cancer metastases, Frontiers in Bioscience 16, 531-538, January 1, 2011

428. Yang et al, Oncolytic Adenovirus, a New Treatment Strategy for Prostate Cancer, Biomedicines 2022, 10, 3262.

429. Yang et al, Role of Exosomes in Crosstalk Between Cancer-Associated Fibroblasts and Cancer Cells, Frontiers in Oncology, May 2019 | Volume 9 | Article 356

430. Yang, J., et al, Activation of FOXO3 is Sufficient to Reverse Mitogen Activated Protein/Extracellular Signal, Can Res, 2010 pp 4709-4718.

431. Ye, Y., et al, Progressive Chromatin Repression and Promoter Methylation of CTNNA1 Associated with Advanced Myeloid Malignancies, Cancer Res Nov 2009.

432. Yi, M., E. Ruoslahti, A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis, pp 620–624, PNAS, January 16, 2001, vol. 98, no. 2.

433. Yin et al, Recognition of self and altered self by T cells in autoimmunity and allergy, Protein Cell 2013, 4(1): 8–16

434. Yu, The Function of NK Cells in Tumor Metastasis and NK Cell-Based Immunotherapy, cancers, 2023

435. Yun et al, Fibroblast Growth Factors: Biology, Function, and Application for Tissue Regeneration, Journal of Tissue Engineering, Volume 2010, Article ID 218142, 18 pages

436. Zak, D., Continuous Time Identification of Gene Expression Models, Journal of Integrative Biology, Vol 7 No 4 2003 pp 373-386.

437. Zamai, L. et al, NK Cells and Cancer, J Immunol 2007; 178:4011-4016.

438. Zent, R., A., Pozzi, Cell-Extracellular Matrix Interactions in Cancer, Springer (New York) 2010.

439. Zhang et al, Bone marrow mesenchymal stem cell-derived exosomes prevent osteoarthritis by regulating synovial macrophage polarization, Aging 2020, Vol. 12, No. 24

440. Zhang et al, Function of Macrophage in Disease: Current understanding on Molecular Mechanisms, Frontiers in Immunology, March 2021

441. Zhang et al, Macrophages regulate the progression of osteoarthritis, Osteoarthritis and Cartilage 28 (2020) 555e561

442. Zhang et al, mRNA vaccines in disease prevention and treatment, Signal Transduction and Targeted Therapy, 2023.

443. Zhang et al, Regulation of epithelial-mesenchymal transition by tumor-associated macrophages in cancer, Am J Transl Res 2015;7:1699-1711

444. Zhang, et al, Prognostic Significance of Tumor-Associated Macrophages in Solid Tumor: A Meta-Analysis of the Literature, PLOS One, Dec 2012

445. Zhao et al, Bladder cancer cells re-educate TAMs through lactate shuttling in the microfluidic cancer microenvironment, Oncotarget, Vol. 6, No. 36 2015

446. Zhu et al, Epithelial derived CTGF promotes breast tumor progression via inducing EMT and collagen I fibers deposition, Oncotarget, Vol. 6, No. 28, 2015

447. Ziani et al, Alteration of the Antitumor immune Response by Cancer-Associated Fibroblasts, Frontiers in Immunology, March 2018, Volume 9, Article 414 Aaronson, Growth Factors and Cancer, Science, Vol 254 Nov 1991.

448. Zingales et al, Metformin: A Bridge between Diabetes and Prostate Cancer, Frontiers in Oncology, October 2017, Volume 7, Article 243

449. Zoete et al, Inflammasomes, Cold Spring Harb Perspect Biol 2014;6:a016287

10 INDEX