IMMUNOTHERAPY: POSSIBLE DIRECTIONS

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We have seen an expansion in the immunotherapeutic approaches to dealing with cancers. There are approaches in dealing with melanoma and lung cancers which were unheard of a decade ago. The hematopoietic cancers can now be dealt with using CAR-T cells, specifically designed T cells. CIK, cytokine induced killer cells use the patients own NK cells to be strengthened and sent back into the fight. TILs have been doing the same for decades now. This note examines the multiplicity of other possible approaches.

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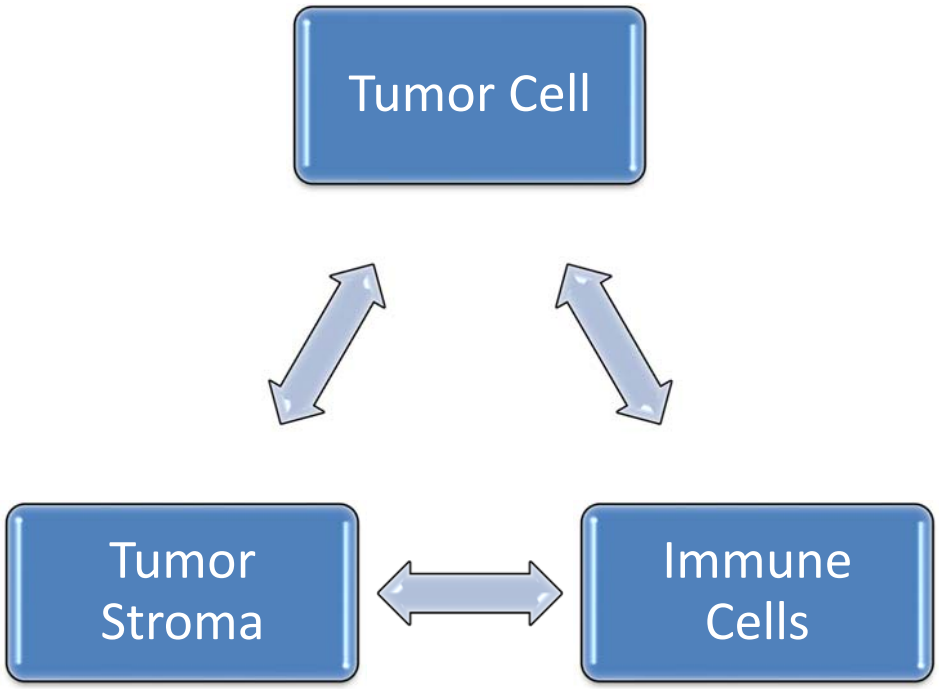
1 INTRODUCTION

The development of the various techniques of immunotherapy is complex and it covers well over a century. The tale of PD-1 alone has been plotted by several and the description by Wang et al is worth noting. The immune system has the ability to attack and destroy aberrant cells, invaders and mutants. This happens frequently but not always. In cancerous growths the immune cells frequently are present but their ability to overcome a cancer is limited.

Discovering what limits the immune cells has been a productive battle. Add to that selective antibodies, monoclonal Ab (MAbs) allows for targeting and suppression or activation of pathways that lead to cancer destruction. Herein we examine some dimensions of this work[1].

Thus, we will examine the issues of the tumor, the immune system and the tumor stroma.



Note that the tumor stroma plays an equally important role. As Bremnes et al have noted:

Maintenance of both normal epithelial tissues and their malignant counterparts is supported by the host tissue stroma. ***The tumor stroma mainly consists of the basement membrane, fibroblasts, extracellular matrix, immune cells, and vasculature.*** *Although most host cells in the stroma possess certain tumor-suppressing abilities, the stroma will change during malignancy*

[1] See the Draft book on Immunotherapy at https://www.researchgate.net/publication/314090163_Cancer_Immunotherapy_A_Systems_Approach

and eventually promote growth, invasion, and metastasis. Stromal changes at the invasion front include the appearance of carcinoma-associated fibroblasts (CAFs). CAFs constitute a major portion of the reactive tumor stroma and play a crucial role in tumor progression. The main precursors of CAFs are normal fibroblasts, and the trans-differentiation of fibroblasts to CAFs is driven to a great extent by cancer derived cytokines such as transforming growth factor. During recent years, the crosstalk between the cancer cells and the tumor stroma, highly responsible for the progression of tumors and their metastasis, has been increasingly unveiled.

A better understanding of the host stroma contribution to cancer progression will increase our knowledge about the growth promoting signaling pathways and hopefully lead to novel therapeutic interventions targeting the tumor stroma. This review reports novel data on the essential crosstalk between cancer cells and cells of the tumor stroma, with an emphasis on the role played by CAFs. Furthermore, it presents recent literature on relevant tumor stroma- and CAF-related research in nonsmall cell lung cancer. …

The tumor stroma basically consists of

(1) the nonmalignant cells of the tumor such as CAFs, specialized mesenchymal cell types distinctive to each tissue environment, innate and adaptive immune cells, and vasculature with endothelial cells and pericytes and

(2) the extracellular matrix (ECM) consisting of structural proteins (collagen and elastin), specialized proteins (fibrilin, fibronectin, and elastin), and proteoglycans[2]

Angiogenesis is central for cancer cell growth and survival and has hitherto been the most successful among stromal targets in anticancer therapy. Initiation of angiogenesis requires matrix metalloproteinase (MMP) induction leading to degradation of the basement membrane, sprouting of endothelial cells, and regulation of pericyte attachment. However, CAFs play an important role in synchronizing these events through the expression of numerous ECM molecules and growth factors, including transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) 2.

We can also call this in certain circumstances the tumor microenvironment, TME.

Historically immune escape was a significant factor. As Prendergast noted:

Immune escape was not widely recognized among cancer geneticists or molecular cell biologists as a fundamental trait of cancer until recently. In the late 1960s, studies of immune deficient nude mice, newly developed at the time, argued that they had no increased susceptibility to spontaneous cancers. An influential interpretation of these findings was that immunity was not a critical restraint to tumorigenesis in mammals.

[2] See https://www.researchgate.net/publication/333704252_EMT_lncRNA_TGF_SMAD_and_Cancers and https://www.researchgate.net/publication/330222973_EMT_and_Cancers also see https://www.researchgate.net/publication/325046881_PCa_mir34_p53_MET_and_Methylation

However, this interpretation was flawed by the lack of knowledge that nude mice retain natural killer cells (NK cells), which have potent antitumor activity. Studies of mutated oncogenes discovered in the late 1970s and 1980s tended to reinforce the notion that immunity was not critical for tumorigenesis, based on demonstrations that malignant cells could be created from normal cells in vitro.

Through the 1990s, the perspective of many cancer geneticists and cancer cell biologists was that unmutated genes had relatively limited roles in cancer pathophysiology, and it was apparent that overt immune regulatory genes were not mutated in cancer. Later, studies in transgenic mouse models and sound clinical documentation of the reality of dormant cancers forced a greater appreciation of how inflammation and immunity contributed to tumorigenesis.

For example, tumors arising in patients who had received a transplant from a donor who years earlier had been cured of cancer were found to be derived from the donor, arguing that occult tumor cells from the transplant could be immunologically managed for long periods as dormant disease until they were moved to an immunosuppressed organ recipient (here, it should be emphasized that the clinical 'cure' achieved in the donor was simply a reduction of the disease to a dormant occult state). In mice, tactics to genetically ablate T-cell function dramatically increased the incidence of spontaneous solid tumors. These findings demonstrated that adaptive immunity performs an essential tumor suppressor function in mammals.

Furthermore, they implied that immune escape is essential for the formation of a tumor. Recent experimental findings directly corroborate the notion that tumor cells can exist in an occult state of immune equilibrium for long periods. In a classical model of chemical carcinogenesis, Schreiber and Smyth and colleagues showed that immune depletion will reveal tumors in mice that will remain tumor free after low doses of carcinogen that are insufficient to trigger tumor formation during the host's lifespan. Evidence of transformed but dormant tumor cells was obtained in animals along with a demonstration that such cells are more immunogenic than those present in frank tumors.

Thus, along with other cell-extrinsic traits of cancer, immune escape is an essential trait for the development of progressive disease, acting as a biological modifier to dictate the outcome of an oncogenically initiated lesion that may otherwise be eliminated or be present in an extended occult state of immune equilibrium corresponding to dormancy.

Here, the definition of a cancer modifier is broadly defined in pathological terms as a gene that is phenotypically silent unless evaluated in the context of cancer. By this definition, many genes influencing cell-extrinsic traits of cancer—angiogenesis, invasion, metastasis and immune escape—are understood as modifiers that dominate tumor outcomes.

In short, while mutations in oncogenes and tumor suppressors start cancer, modifiers and the microenvironment which act later may dictate their clinical relevance. Learning how immune escape evolves during the integrated processes of oncogenesis and immunoediting may therefore yield more powerful insights into cancer pathophysiology and therapy than achieved to date.

Our focus is based upon the papers by Marin-Acevedo et al who provided an excellent systematic survey of various immunotherapeutic approaches. The most well know has been the

PD-1 and CTLA4 antibody approaches facilitating the immune systems in its attack on various cancers. The second dimension is the CAR-T cell approach and the CD19 targets on hematopoietic cancers. In previous papers we also examine the use of CIK, cytokine induced killer cells using the innate system and NK cells[3].

However, it is well known that there are a multiplicity of other well-known ligands and the like as well as various innate system methods and also other cell lines which can attack various malignancies. On top of these there most likely an equal number or more that we have yet to discover. Our objective herein is to follow Marin-Acevedo et al and to use their outline and fill it in with other details while allowing for putative expansion in new markers and ligands.

Our approach is as follows:

1. Examine the Tumor Micro Environment, TME, as a possible and recognized limiting factor. This has been a problem especially with solid tumors. The TME acts as a protective shell and the approaches to exciting the immune system must take this factor into consideration.

2. Examine the most significant players in the immune system. We have often considered the innate as well as the adaptive as essential elements. Just examining T cells, albeit powerful players, may be too delimiting. In addition multiple therapeutics using both lines have significant potential. Also, some of the innate cells may have pro-malignant capabilities so one must beware as well.

3. Alternative approaches such as BiTE and DART are considered. These are Ab, anti-body, manipulated approaches and allow for multiple attacks with the same Ab.

4. Using the Marin-Acevedo et al systematics for characterization we summarize their effort by expanding the description across a differing bases of source material.

5. We end with a set of observations spanning the gamut of current understanding of the multiplicity of approaches still available.

Clearly the immunotherapeutic approaches have just begun to have been examined. The goal here is to try to set forth a systematized approach and a methodology to maintain some currency.

[3] https://www.researchgate.net/publication/280627292_MDS_METHYLATION_AND_THE_EPIGENETIC_PARADIGM

2 TUMOR MICRO ENVIRONMENT

Before considering the attack on cancer cells with immunotherapeutic methods, one must understand that they establish themselves in a protective environment, the tumor microenvironment, TME. The tumor micro-environment (TME) is the complex of interacting aggregate tumor cells. The immune system generally acts to attack and eliminate invaders but the TME has the capability to modify many of these functions. The presence and structure of the TME must be understood for each malignant form so as best to attack with immune cells. If not done so, then the attacking agents may just "bounce off" this protective shell.

As Tredan et al have noted:

Resistance of human tumors to anticancer drugs is most often ascribed to gene mutations, gene amplification, or epigenetic changes that influence the uptake, metabolism, or export of drugs from single cells. Another important yet little-appreciated cause of anticancer drug resistance is the limited ability of drugs to penetrate tumor tissue and to reach all of the tumor cells in a potentially lethal concentration.

To reach all viable cells in the tumor, anticancer drugs must be delivered efficiently through the tumor vasculature, cross the vessel wall, and traverse the tumor tissue. In addition, heterogeneity within the tumor microenvironment leads to marked gradients in the rate of cell proliferation and to regions of hypoxia and acidity, all of which can influence the sensitivity of the tumor cells to drug treatment. In this review, we describe how the tumor microenvironment may be involved in the resistance of solid tumors to chemotherapy and discuss potential strategies to improve the effectiveness of drug treatment by modifying factors relating to the tumor microenvironment. ...

The above is a critical factor. Tumor cells seem to develop a significant amount of defense mechanisms. As we will note latter, they even turn the immune system against itself, using macrophages to feed the tumor and block T cell attack. They continue:

Solid tumors are organ-like structures that are heterogeneous and structurally complex. They comprise cancer cells and stromal cells (i.e., fibroblasts and inflammatory cells) that are embedded in an extracellular matrix and nourished by a vascular network; each of these components may vary from one location to another in the same tumor.

The TME is thus the complex assembly of the cells, the ECM, the vasculature and its arrangement and cohesiveness. The TME is a complex infrastructure which tends to protect the mass of cancer cells and this protection allows them to proliferate while being protected against any of the attempt of the immune system to battle them. The authors continue:

The effectiveness of drug therapy is impaired by limited delivery of drugs to some regions of tumors and by effects of the tumor microenvironment on drug activity and on the metabolism and proliferation of tumor cells. Agents that improve drug delivery or activity by targeting the tumor microenvironment, especially in hypoxic regions of tumors, represent an important future direction for cancer therapy.

Adding vascular-disrupting agents that increase the extent of the hypoxic/acidic region might enhance the anticancer activity of various drugs that show increased efficacy against acidic cells, hypoxia-activated prodrugs, or bacteriolytic therapies. The development of methodologies to characterize causes of drug resistance related to the tumor microenvironment has considerable potential to improve the outcomes of patients following systemic treatment of solid tumors.

The same effects to drug therapy will apply to immunotherapy and must be addressed accordingly. Nyberg et al further note:

The tumor microenvironment is a mixture of extracellular matrix molecules, tumor cells, endothelial cells, fibroblasts and immune cells. Tumor growth and metastasis formation are dependent on the growth of blood vessels into the tumor mass. The tumor microenvironment contributes to this pathological angiogenic process.

The extracellular matrix and basement membranes are a source for endogenous angiogenesis inhibitors, such as endostatin. On the other hand, many extracellular matrix molecules can promote angiogenesis by stabilizing blood vessels and sequestering pro-angiogenic growth factors. The majority of stromal cells in carcinomas are fibroblasts. Carcinoma- associated fibroblasts show a distinct phenotype from normal fibroblasts.

The mechanisms how the tumor- associated fibroblasts regulate angiogenesis are not fully known, but they are suggested to be an important source for growth factors and cytokines recruiting endothelial cells. The immune cells, particularly macrophages and neutrophils are another source for angiogenesis-regulating chemokines, growth factors and proteases. Taken together, the tumor microenvironment is a complex unorganized tissue of various cell types and extracellular matrix that can regulate the pathological angiogenic switch.

Zhang et al have recently described various technique as how to modify the TME so as to be more accepting of immunotherapy. As Bassani et al note:

Strong evidences suggest that the presence of inflammatory cells within the TME plays a crucial role in the development and/or progression of tumors. Among the host-dependent biological features of the tumor hallmarks defined by Hanahan and Weinberg, there are "evading immune destruction" and "tumor-promoting inflammation", which together with the immune cell-mediated orchestration of angiogenesis, point out the key role of the immune system in neoplastic disease.

As a consequence of their functional plasticity, several immune cells, can modify upon stimuli delivered by the components of TME their phenotypic and functional features; this leads to a reduced killing of tumor cells, the expression of a tolerogenic/immunosuppressive behavior and the acquisition of pro-angiogenic activities, thus promoting tumor expansion. NK cells are innate lymphocytes that can potentially control tumor growth by their cytotoxic activity.

The plasticity is a key factor for hiding itself from normal immune response mechanisms. They continue:

Classical NK cells are distinct from innate lymphoid cells (ILCs) although they share with ILC1 several phenotypic features; indeed, NK cells are key cytolytic effectors of innate immunity while ILC1 are generally non-cytotoxic or weakly cytotoxic but they show a central role in response to certain infections and are also involved in tissue remodeling homeostasis, morphogenesis, metabolism, repair, and regeneration. ....

ILC and NK cells originate from a common lymphoid progenitor (CLP). GATA3 or TOX/NFIL3/ID2/ETS1 drive the distinction between common innate lymphoid progenitor (CLIP) and the NK cell progenitor (NKP), respectively. Finally, T-bet/EOMES expression in NKPs govern NK cell differentiation. Natural killer cell subsets can differ according to tissue distribution that is related to distinct homing properties and/or local maturation

These environments also provide a base for various growth factors. Bremnes et al list the following table for the key growth factors that control, sustain and enable metastatic expansion via the blood stream:

Factor	Function
TGF-α	Considered important in wound healing. Induces epithelial development. Closely related to EGF.
TGF-β	TGF- is the most frequent. Normally controls proliferation and cellular differentiation. Role in immunity and cancer. In cancer, it may lead to progression and metastasis.
PDGF	Promotes proliferation of connective tissue. Regulate cell growth and division. Role in angiogenesis. Regulates interstitial fluid pressure.
FGF2	Present in BM and in the subendothelial ECM of vessels. Promotes proliferation of different cells. Role in angiogenesis.
EGF	Binds to its receptor EGFR, leads to cellular proliferation, differentiation, and survival.
VEGF	Increase vascular permeability. Role in early stages of desmoplasia. Important role in angiogenesis.
HGF	Paracrine cellular growth, motility, and morphogenic factor. Secreted by mesenchymal cells, acts on epithelial or endothelial cells.
IGF-1	Growth factor. Has growth-promoting effect on almost every cell in the body.

Factor	Function
CTGF	Can promote endothelial cell growth, migration, adhesion, and survival. It is implicated in endothelial cell function and angiogenesis.
CXCLs and CCLs	Chemokines of the CXC and CL types. Attractants of leukocytes. Important in angiogenesis, carcinogenesis, tumor progression, and metastasis.
SFRP1	Act as soluble modulators of Wnt signaling.
SPARC	Associated with cancer cell migration and invasion.
ILs	Cytokines. Inflammatory response against infection. Enables transmigration of lymphocytes. IL-1 and -6 may contribute to cancer progression.

These growth factors will become major supports for the tumor cells as well.

3 KEY IMMUNE SYSTEM CELLS

We now provide a brief summary of some of the key immune system cell types. It should be noted as these cells are examined over time further classifications are made and specific functions of these new groups are identified. I believe it is fair to state that we shall learn a great deal more about the subtleties of the immune system as time goes by. For many the understanding of PD-1 and CTLA4 appear to be an end point but the key observation in this note it to demonstrate that they are perhaps just the beginning.

3.1 MAST CELLS

Mast cells are major effector cell of immediate hypersensitivity (allergic) reactions. Mast cells are derived from the marrow, reside in most tissues adjacent to blood vessels, express a high-affinity Fc receptor for IgE, and contain numerous mediator-filled granules. Antigen-induced cross-linking of IgE bound to the mast cell Fc receptors causes release of their granule contents as well as new synthesis and secretion of other mediators, leading to an immediate hypersensitivity reaction[4]. As Varricchi et al have noted:

Mast cells were first identified in human tumors and named by Paul Ehrlich. These cells are present in all classes of vertebrates, and it has been estimated that they have emerged >500 million years ago, long before the development of adaptive immunity. Mast cells are distributed throughout nearly all human tissues and often in close proximity to epithelia, fibroblasts, blood and lymphatic vessels, and nerves.

Human mast cells form a heterogeneous population of cells with differences in their ultrastructure, morphology, mediator content, and surface receptors. Human mast cells derive from CD34+, CD117+ pluripotent hematopoietic stem cells, which arise in the bone marrow. Mast cell progenitors enter the circulation and subsequently complete their maturation in tissues. These cells store and release upon activation a wide spectrum of biologically active mediators that individually have been shown to have potential positive or negative effects on various target cells.

Mast cells can be the source of the cytokines that if are produced in excess can result in significant cellular damage. They continue:

Increasing evidence indicates that mast cells act as sentinels of the surrounding environment, with the capacity to rapidly perceive tissue insults and initiate biochemical programs of inflammation or repair. Mast cells are activated not only by IgE, specific antigens, and superallergens, the main mechanisms which account for their function in allergic disorders, but also by a plethora of immunologic and non-immunologic stimuli …

(there is a) constellation of surface receptors expressed by human mast cells. Mast cells and their mediators have been canonically associated with a detrimental role in allergic diseases, but

[4] See Abbas et al 4th Ed

these cells can induce a protective immune response of the host against noxious substances, viral and microbial pathogens. Interestingly, epidemiological and experimental studies indicate an inverse association between IgE-mediated allergies and cancer, implying tumor-protective effect of IgE.

Mast cells can thus be considered as sentinels which reside in tissue at the ready to attack and intruder.

3.2 NK

Natural killer cells, NK, are elements of the innate immune system. 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[5].

From 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****) 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.*

The KIR receptors will play an important role as we discuss latter. The authors continue:

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

[5] See Abbas et al 4th Ed

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. 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.

3.3 NKT

The NK T cell is neither a CTL nor an NK cell. It is a third variety somewhat in between. CTL are adaptive and NK are innate. The T cell receptor on NKT cells does not recognize MHC molecules and it has markers similar to both NK and CTL. Natural killer T cells (NK-T cells) are a numerically small subset of lymphocytes that express some of the T cell receptors and some surface molecules characteristic of NK cells. Some NK-T cells, called invariant (iNK-T), express αβ T cell antigen receptors with minimal diversity, recognize lipid antigens presented by CD1 molecules, and perform various effector functions typical of helper T cells.

As Ibarrondo et al note that there is a group of NKT cells called "invariant" and are described as follows:

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

From Kumar et al we have further descriptions of NKT cells as follows:

Type I natural killer T (NKT) cells are innate-like T lymphocytes that recognize glycolipid antigens presented by the MHC class I-like protein CD1d. Agonistic activation of NKT cells leads to rapid pro-inflammatory and immune modulatory cytokine and chemokine responses. This property of NKT cells, in conjunction with their interactions with antigen-presenting cells, controls downstream innate and adaptive immune responses against cancers and infectious diseases, as well as in several inflammatory disorders. NKT cell properties are acquired during development in the thymus and by interactions with the host microbial consortium in the gut, the nature of which can be influenced by NKT cells. This latter property, together with the role of the host microbiota in cancer therapy, necessitates a new perspective.

They continue regarding NKT cells:

NKT cells—originally defined as cells that co-express key natural killer (NK) cell surface markers and a conserved αβ TCR repertoire—are thymus-derived, innate-like T lymphocytes.

The functions of NKT cells are controlled by self and non-self-lipid agonists presented by CD1d molecules. The majority of NKT cells (type I, invariant NKT) express an invariant TCR α-chain (Vα14Jα18 in mice; Vα24Jα18 in humans). The invariant α-chain pairs predominantly with Vβ8.2, Vβ7, or Vβ2 in mouse NKT cells, or Vβ11 almost exclusively in human NKT cells. A small NKT cell population—referred to as type II NKT cells—expresses a more diverse TCR repertoire and recognizes a distinct group of lipid antigens; these, however, are the focus of other reviews.

The recognition of lipid agonists rapidly activates NKT cells, which respond just as quickly by secreting a variety of cytokines and chemokines, and upregulate costimulatory molecules. By acting promptly, NKT cells alert and regulate the effector functions of myeloid and lymphoid cells. In so doing, NKT cells play a critical role in controlling microbial and tumor immunity as well as autoimmune and inflammatory diseases

3.4 CTL OR KILLER T CELLS

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

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

As Steer et al note:

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

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

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

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

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

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

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

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

3.5 CIK

Cytokine induced killer cells, CIKs, are an exogenously made set of NK cells which have been grown in vitro from cells taken from the patient and induced by cytokines. As Introna and Correnti have noted:

Cytokine-induced killer (CIK) cells are T lymphocytes that have acquired, in vitro, following extensive manipulation by Interferon gamma (IFN-γ), OKT3 and Interleukin 2 (IL-2) addition, the expression of several Natural Killer (NK) cell-surface markers. CIK cells have a dual "nature", due to the presence of functional TCR as well as NK molecules, even if the antitumoral activity can be traced back only to the NK-like structures (DNAM-1, NKG2D, NKp30 and CD56).

In addition to antineoplastic activity in vitro and in several in-vivo models, CIK cells show very limited, if any, GvHD toxicity as well as a strong intratumoral homing. For all such reasons, CIK cells have been proposed and tested in many clinical trials in cancer patients both in autologous and allogeneic combinations, up to haploidentical mismatching. Indeed, genetic modification of CIK cells as well as the possibility of combining them with specific monoclonal antibodies will further expand the possibility of their clinical utilization. Cytokine-induced killer (CIK) cells are non-MHC (Major Histocompatibility Complex) restricted, cytotoxic antitumoral cells expanded in vitro from circulating precursors. CIK cells share characteristics of both T and NK cells.

We have previously demonstrated how these cells have been used to eliminate MDS in certain patients[6]. The CIK cells can be well targeted, well tolerated, and quite effective.

[6] https://www.researchgate.net/publication/280627292_MDS_METHYLATION_AND_THE_EPIGENETIC_PARADIGM

Based on the published results obtained both in vitro and in vivo and with cells of both mouse and human origin, CIK cells show, in vivo, a very strong cytolytic activity against leukemia and graft versus leukemia (GVL), while being essentially devoid of graft-versus-host reactivity (GvHD). Indeed, it has long been known that cytotoxic cells with this double T/NK phenotype are rare but present (from 1% to 5%) in circulating blood … and are capable of lysing a broad array of tumor cell targets in a non-MHC-restricted manner.

3.6 TIL

Tumor infiltrating lymphocytes have been known for a few decades and have been used as a means to attack melanoma cells. From Abbas et al:

TILs are lymphocytes isolated from the inflammatory infiltrates present in and around surgical resection samples of solid tumors that are enriched with tumor-specific CTLs and NK cells. In an experimental mode of cancer treatment, TILs are grown in vitro in the presence of high doses of IL-2 and are then adoptively transferred back into patients with the tumor.

As Matsutani et al have noted:

As the primary host immune response against malignant tumors, tumor-infiltrating lymphocytes (TILs) have been reported to have a crucial effect on tumor progression and the clinical outcome in various types of cancer, including non-small cell lung cancer (NSCLC), colorectal, esophageal, and urothelial cancers and melanoma. Furthermore, … reported that the density of TILs are more valuable prognostic markers than the TNM classification. However, while a number of methods have been proposed for evaluating the density of TILs, none has yet been confirmed to be optimum.

TILs have been used in various cases but they do not seem to be as well targeted as other means. From Horton and Gajewski:

Tumours from multiple cancer types can be infiltrated by CD8þ T cells (TILs). TILs are thought to be suppressed by multiple immune inhibitory molecules in the tumour microenvironment, and this suppression has been associated with tumour progression. Therefore, despite tumour infiltration, almost all tumours containing TILs will progress if not treated. While several immune inhibitory mechanisms have been identified, immune inhibitory receptors expressed on activated T cells, like CTLA-4 and PD-1, have received the most attention over recent years owing to the immense clinical success of PD-1 and CTLA-4 neutralising antibodies.

The engagement of inhibitory receptors expressed by TILs is thought to render TILs dysfunctional. However, evidence from both human tumour samples and mouse models has suggested that, despite inhibitory receptor expression, TILs are not functionally inert and actually retain the ability to proliferate, produce IFN-g, and show ex vivo cytotoxicity. These observations raise the question of why activated TILs are not able to spontaneously control progressing tumours, and how tumours that contain TILs might sometimes be resistant to immunotherapies such as checkpoint blockade.

Current immunotherapies can induce durable tumour regression; however, they benefit a minority of patients: finding new strategies to increase the response rate to immunotherapies is of great interest to both researchers and clinicians.

3.7 MACROPHAGES

Macrophages are ubiquitous and generally are supportive members of the immune system. From Abbas et al:

Macrophage Tissue-based phagocytic cell derived from blood monocytes that plays important roles in innate and adaptive immune responses. Macrophages are activated by microbial products such as endotoxin and by T cell cytokines such as IFN-γ. Activated macrophages phagocytose and kill microorganisms, secrete proinflammatory cytokines, and present antigens to helper T cells. Macrophages may assume different morphologic forms in different tissues, including the microglia of the central nervous system, Kupffer cells in the liver, alveolar macrophages in the lung, and osteoclasts in bone.

However, macrophages may also play a tumor enhancing role in some cancers. As Lewis and Pollard have noted:

Macrophages are prominent in the stromal compartment of virtually all types of malignancy. These highly versatile cells respond to the presence of stimuli in different parts of tumors with the release of a distinct repertoire of growth factors, cytokines, chemokines, and enzymes that regulate tumor growth, angiogenesis, invasion, and/or metastasis. The distinct microenvironments where tumor-associated macrophages (TAM) act include areas of invasion where TAMs promote cancer cell motility, stromal and perivascular areas where TAMs promote metastasis, and avascular and perinecrotic areas where hypoxic TAMs stimulate angiogenesis. This review will discuss the evidence for differential regulation of TAMs in these microenvironments and provide an overview of current attempts to target or use TAMs for therapeutic purposes.

There are certain cancers where the macrophage presence bodes well such as melanomas and on the otherhand it bodes poorly in uveal melanoma. The authors continue:

The roles of different subpopulations of TAMs in tumor progression.

1, invasion: TAMs secrete a variety of proteases to breakdown the basement membrane around areas of proliferating tumor cells (e.g., ductal carcinoma in situ in the breast), thereby prompting their escape into the surrounding stroma where they show deregulated growth.

2, angiogenesis: In areas of transient (avascular) and chronic (perinecrotic) tumor hypoxia, macrophages cooperate with tumor cells to induce a vascular supply for the area by up-regulating a number of angiogenic growth factors and enzymes. These diffuse away from the hypoxic area and, together with other proangiogenic stimuli in the tumor microenvironment, stimulate endothelial cells in neighboring, vascularized areas to migrate, proliferate, and differentiate into new vessels.

3, immunosuppression: Macrophages in hypoxic areas secrete factors that suppress the antitumor functions of immune effectors within the tumor.

4, metastasis: A subpopulation of TAMs associated with tumor vessels secretes factors like EGF to guide tumor cells in the stroma toward blood vessels where they then escape into the circulation.

In the stromal compartment (both the acellular regions and others where they are in close contact with tumor cells), TAMs secrete growth factors to stimulate tumor cell division and/or undefined factors that promote tumor cell motility.

We shall come back to this again but the point worth noting is that macrophages when combined in the TME can actually become supportive of the tumor itself. It thus turns on its host.

3.8 DENDRITIC CELLS

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

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

As Lubong and Bhardwaj (Nature 2015) note:

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

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

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

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

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

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

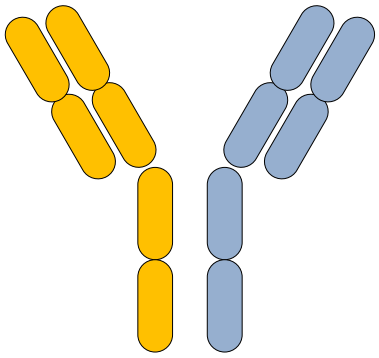
From Abbas et al:

Dendritic cells (DCs) are bone marrow–derived cells found in epithelial and lymphoid tissues that are morphologically characterized by thin, membranous projections. Many subsets of DCs exist with diverse functions. Activated (mature) DCs function as antigen presenting cells (APCs) for naive T lymphocytes and are important for initiation of adaptive immune responses to protein antigen. Immature (resting) DCs are important for induction of tolerance to self-antigens.

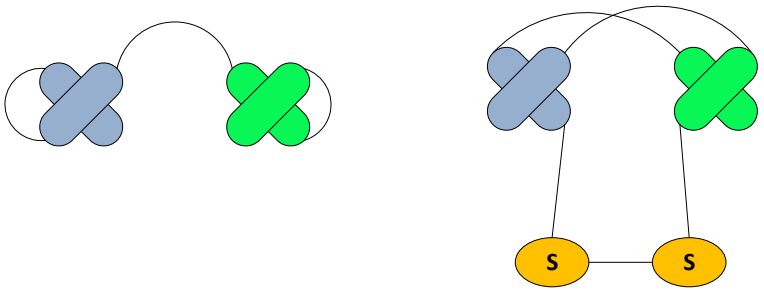
Dendritic cells as modified have been used as a targeting entity for certain immunotherapy approaches.

4 SOME OTHER APPROACHES

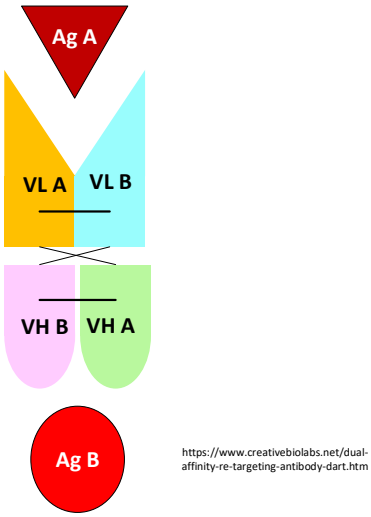
Developing antibodies to effect the control of T cells and the like has been extended in various ways. Abs can now be "manufactured" and implemented to effect a variety of tasks. The figure below is a classic Ab structure with long and short segments.



Now there are two variations, amongst many, that can be designed to perform the activation of T cells; the BiTE and DART designs. As shown below the BiTE and DART designs take segments of the Ab and connect them together in a variety of ways. BiTE via cross linking and DART with both crosslinking and a di-sulfide bond.



The figure below details the DART approach showing the ability to deal with two antigen bonding capabilities.



We will now provide some details on these two options.

4.1 BiTE

We now discuss the BiTE approach and we start with the comments by Choi et al who note:

Bispecific antibodies were first developed upon previously established principles of monoclonal antibody therapy -- namely that, in the treatment of malignant diseases, antibodies had already been shown to possess the specificity necessary to mediate a number of antigen-specific immune mechanisms.

Native antibodies of the IgG type for example are made up of two identical, antigen-binding, variable regions joined by a constant fragment domain (Fc). Monocytes and other phagocytic cells bind Fc domains via their surface Fcg receptors, resulting in specific lysis of targeted cells through a well-characterized antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism. Besides interacting with cellular effectors however, antibodies also recruit endogenous proteins of the complement cascade, or alternatively, they can be artificially modified to deliver a payload of cytotoxic molecules including radioisotopes, chemotherapies or bacterial toxins to tumors with great specificity.

Like armed monoclonal antibodies, bispecific antibodies do not occur naturally in the human body and must be created using either recombinant DNA or cell-fusion technologies.

These techniques make it possible to combine multiple humoral specificities into a single molecule while retaining the specificity -- and occasionally the function -- of each component contributing to its overall design. Thus, in the most general sense, the term 'bispecific antibody' refers to a class of constructs in which two antibody-derived antigen-specific binding sites are aligned within one molecule. In the context of tumor immunotherapy, this typically means that one arm of the construct is specific for an epitope on the surface of a cancer cell (target-binding arm), while the other arm is specific for the effector cell (effector-binding arm).

The theoretical benefit of this divalent design lies in its potential to simultaneously redirect and locally activate cellular effectors in the presence of cancer cells, thereby maximizing proximate target lysis while minimizing non-specific cytotoxicity in surrounding healthy cells and tissues. The concept of using bispecific antibodies to activate T cells against tumor antigen was first described over 20 years ago.

Among the first formats pursued were constructs designed to bind the monomorphic TCR--CD3 complex, a strategy that offers a number of conceivable advantages. Triggering lysis by this approach allows bispecific antibodies to interact globally with the T cell compartment, thereby circumventing the restriction of clonotypic specificity and proliferation. In addition, because the target-binding arm is derived at least in part from the variable portion of a tumor-specific antibody, the overall effect is not only highly specific, but also widely applicable against a broad array of antigens -- that is, antibodies possess the ability to bind tumor epitopes beyond the MHC-peptide complexes classically recognized by TCR.

As a result, bispecific antibodies both increase the complement of targetable T cell antigens and also overcome mechanisms of tumor immune escape such as loss or downregulation of MHC.

From a different perspective, Ross et al note have noted about the BiTE approach:

BiTE antibody constructs comprise tandemly-arranged single-chain variable fragments (scFvs). One scFv binds the TCR CD3ε subunit and the other binds a ***tumor-associated surface antigen (TAA)****.*

BiTE antibody constructs have been shown to induce the formation of a cytolytic synapse between the T cell and the transiently-linked tumor cell.

Target cell lysis occurs in the absence of regular major histocompatibility complex (MHC) class I/peptide antigen recognition and costimulation, and is therefore resistant to certain immune escape mechanisms affecting antigen presentation and those affecting generation of tumor-specific T cell clones.

T cell activation by BiTE1 antibody constructs ***is strictly dependent on the presence of cells expressing the TAA****. Because the CD3ε target of BiTE antibody construct is invariant, both CD8+ and CD4+ T cells of any phenotype can be engaged, leading to a polyclonal T cell activation, expansion and tumor cell lysis*

Finally Goswami et al have noted:

Bispecific, monovalent antibodies were first described in 1961 by Nisonoff and Rivers. The binding of at least two molecular targets with one single bispecific antibody (bsAb) is an attractive therapeutic concept. Bispecific compounds are being developed to enable:

(1) simultaneous inhibition of two cell surface receptors;

(2) simultaneous blocking of two ligands;

(3) cross linking of two receptors; and

(4) recruitment of T-cells to the proximity of tumor cells; to name a few.

The formats currently employed include tandem single chain Fv (scFv), diabodies, tandem diabodies, dual variable domain antibodies, and hetero-dimerization.

There have been multiple attempts to implement BiTE protocols.

4.2 DART

DART is in a sense a slightly more complex design as we have shown previously. As Marin-Acevedo et al note:

DART consists of a diabody that separates variable domains of heavy and light chains of the two antigen-binding specificities on two separate polypeptide chains stabilized through a C-terminal disulfide bridge which acts as a linker.

Compared with BiTE, DART has shown a moderately higher association rate constant for CD3 and an ability to cross-link T cells and B cells more efficiently. Ongoing clinical trials will provide more insightful understanding through side-by-side comparison of DART, BiTE, and other bispecific antibody with identical antigen-binding specificities. The quality, stability, and drug distribution of antibodies remain a challenge.

From the vendor[7]:

Bi-single domain antibody consists of two VH domains linked by a hinge. It is the simplest form of bispecific antibody (BsAb). The small size of bi-single domain antibody endows it incomparable capability in tissue penetration, which makes it an effective tool for delivering therapeutic molecules or effector cells accurately.

However, the minimalized structure constrains the necessary conformational flexibility during antibody-antigen recognition, which in turn reduces the binding efficiency.

Therefore, DARTs are developed to solve this problem. A DART molecule is consisted of two engineered Fv fragments which have their own VH exchanged with the other one. In detail, the Fv1 is consisted of a VH from antibody A and a VL from antibody B, while the Fv2 is consisted of VH from Ab-B and VL from Ab-A. This inter-exchange of Fv domains releases variant fragments from the conformational constraint by the short linking peptide. It ideally mimics the natural interaction within an IgG molecule.

Furthermore, DART molecules are also resistant to aggregation during frozen storage and are potent both in in vitro and in vivo administration. Due to the small size and rapid renal elimination in vivo, DARTs are still on its way to be fully developed, especially for chronic

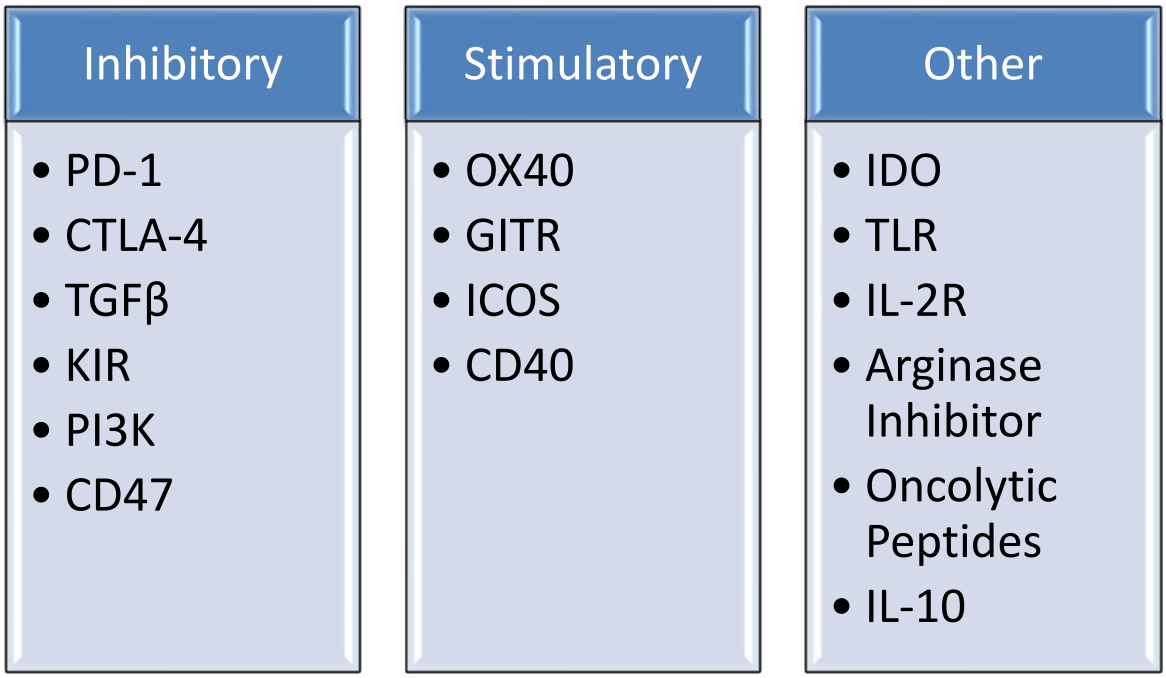
[7] https://www.creativebiolabs.net/dual-affinity-re-targeting-antibody-dart.htm

disease treatment. However, fusing DART to an Fc region can significantly prolong its serum half-life, which gives physicians a broad range for choice in dosing.

These are but two of the possibilities. As the "tools" evolve, we would expect a significant increase in special designs.

5 CATEGORIZATION

We examine several pathways that impact the actions of the immune system. They are inhibitory, stimulatory and other. We depict several of these in the figure below.



5.1 INHIBITORY PATHWAYS

The inhibitory pathways include such ad PD-1 and CTLA-4. Unless blocked they can inhibit the action of the immune system in attacking the cancer cells[8]. Current interest is on these two for now clinical application but there are a multiplicity of other such inhibitory pathways as well.

5.1.1 T Cell Associated

Some of these inhibitory target T cells. LAG-3 for example can inhibit both T cells and NK cells. LAG-3 is often co-expressed along with PD-1. Thus, it is essential in examining the efficacy of many of these inhibitor blocking mechanisms to note that their lack of function is not a lack of operating but the presence of other blockers. Marin-Acevedo presents details on these as we do in the ending section.

5.1.2 Non-T Cell Associated; TGF-β

[8] https://www.researchgate.net/publication/315374574_PD-1_ANOTHER_IMMUNE_INHIBITION_FOR_T_CELL_THERAPEUTICS_FOR_MELANOMA

Transforming Growth Factor β is one of a multiplicity of powerful growth factors. These GF have powerful impacts on cells and their over expression is often found in malignant environments. Fundamentally the GF is produced by a source cell and then is attached to a target cell via a receptor and from that a cascade of response ensue.

As Morikawa et al (2016) have noted there are 33 known human TGF polypeptides. There are 3 TGF-β isoforms, BMP or bone morphogenetic proteins, 10 of them, growth and differentiation factors, GDPs, some 10 of them, some inhibins (5), Mullerian inhibiting substance, and Lefty A and B, Nodal, and myostatin. These ligands bind to a set of receptors and the result in a set of various useful and at time deleterious cellular actions. TGF-β are also known to regulate lncRNAs as we have seen herein. Also, TGF-β is known to be an active driver of EMT.

TGF is a family and its most well understood member is TGF-β1. To best understand its functioning, we must first understand its synthesis and activation and then as it migrates in the extracellular matrix, ECM, its impact on other cells. We first consider activation.

5.1.2.1 TGF Activation

The activation elements are best described by Kubiczkova et al:

Mature dimeric form of TGF-β, composed of two monomers stabilized by hydrophobic interactions and disulphide bridge, initiates intracellular signaling. The three TGF-βs are synthesized as pro-proteins (pro-TGF- βs) with large amino-terminal pro-domains (called latency associated proteins – LAPs), which are required for proper folding and dimerization of carboxy-terminal growth-factor domain (mature peptide).

This complex is called 'small latent complex' (SLC). After folding and dimerization, TGF-β dimer is cleaved from its propeptides in trans-Golgi apparatus by furin type enzymes; however, it remains associated with its pro-peptide through noncovalent interactions, creating 'large latent complex '(LLC). Most cultured cell types release latent TGF-β into extracellular matrix as LLC which in addition includes a 120–240 kDa glycoprotein called latent TGF-β binding protein (LTBP) [24]. LTBP is composed primarily of two kinds of cysteine-rich domains: EGF-like repeats (most of which are calcium-binding) and eight-cysteine domains.

LTBP participates in the regulation of latent TGF-β bioavailability by addressing it to the extracellular matrix (ECM). Nonactive TGF-β stays in ECM; its further activation is a critical step in the regulation of its activity. A number of papers have reported TGF-β activation by retinoic acid and fibroblast growth factor-2 (FGF-2) in endothelial cells, or by endotoxin and bleomycin in macrophages.

Further, a variety of molecules is involved in TGF-β activation. Proteases including plasmin, matrix metaloproteases MMP-2 and MMP-9, are TGF-β activators in vitro. Other molecules involved in the mechanism of activation are thrombospondin-1, integrins, such as αVβ6 or αVβ8, or reactive oxygen species (ROS). Moreover, latent TGF-β present in conditional medium is activated by acid treatment (pH 4.5) in vitro. In vivo, a similar pH is generated by osteoclasts

during bone resorption. Since the bone matrix deposited by osteoblasts is rich in latent TGF-β, the acidic environment created by osteoclasts in vitro might result in latent TGF-β activation.

The TGF comes out of the producing cell as a dimer and then works its way through the ECM. The authors continue:

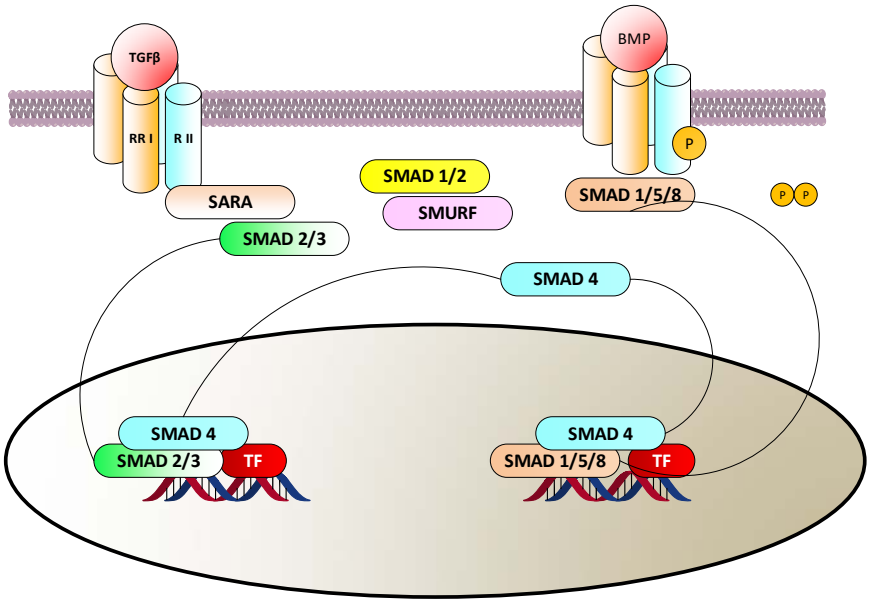
TGF-βs are synthesized as inactive precursors that contain pre-region (Signal peptide) and pro-region (N terminal peptide - LAP). Processing of inactive form starts with proteolytic cleavage that removes signal peptide from pre-pro-TGF-βs form. After dimerization, TGF-βs are cleaved by proteases (eg. Furin) into C-terminal mature peptides and N-terminal LAP (Latency Associated Peptide). TGF-βs with LAP form small latent complexes (SLP) that are transported to extracellular matrix where can further covalently bind to latent TGF-β binding protein (LTBP) to form a large latent complexes (LLC). LTBP is able to connect inactive TGF-β forms to ECM proteins.

This interaction is further supported by covalent transglutaminase-induced (TGase) crosslinks. Activation of TGF-β starts with release of LCC from ECM by proteases. Then, the mature protein is cleaved from LTBP, which is provided in vitro by acidic condition, pH or plasmin or in vivo by thrombospondin (TSP). Once the active TGF-β family member is released from the ECM, it is capable of signaling.

When the TGF gets to a cell with an appropriate and activated receptor set then it can initiate the SMAD pathways for internal cellular actions. We consider these next.

5.1.2.2 TGF Pathways

Once the TGF has been produced it finds its way to a target cell with an appropriate receptor, composed of a complex of Type I and II dimers. From Cantley et al we present a slightly modified TGF/SMAD interaction. We demonstrate two of the TGF actions as shown below:



Now TGFβ is described in NCBI as follows[9]:

This gene encodes a secreted ligand of the TGF-beta (transforming growth factor-beta) superfamily of proteins.

Ligands of this family bind various TGF-beta receptors leading to recruitment and activation of SMAD family transcription factors that regulate gene expression.

The SMAD transcription factor then is activated and its response leads to many of the resulting malignant changes. We shall review SMAD in the following section. NCBI continues:

The encoded preproprotein is proteolytically processed to generate a latency-associated peptide (LAP) and a mature peptide, and is found in either a latent form composed of a mature peptide homodimer, a LAP homodimer, and a latent TGF-beta binding protein, or in an active form consisting solely of the mature peptide homodimer. The mature peptide may also form heterodimers with other TGFB family members.

This encoded protein regulates cell proliferation, differentiation and growth, and can modulate expression and activation of other growth factors including interferon gamma and tumor necrosis factor alpha. This gene is frequently upregulated in tumor cells, and mutations in this gene result in Camurati-Engelmann disease.

As Derynck et al have noted:

Epithelial and hematopoietic cells have a high turnover and their progenitor cells divide continuously, making them prime targets for genetic and epigenetic changes that lead to cell transformation and tumorigenesis. The consequent changes in cell behavior and responsiveness result not only from genetic alterations such as activation of oncogenes or inactivation of tumor suppressor genes, but also from altered production of, or responsiveness to, stimulatory or inhibitory growth and differentiation factors.

Among these, transforming growth factor β (TGF-β) and it signaling effectors act as key determinants of carcinoma cell behavior. The autocrine and paracrine effects of TGF-β on tumor cells and the tumor micro-environment exert both positive and negative influences on cancer development. Accordingly, the TGF-β signaling pathway has been considered as both a tumor suppressor pathway and a promoter of tumor progression and invasion. Here we evaluate the role of TGF-β in tumor development and attempt to reconcile the positive and negative effects of TGF-β in carcinogenesis.

Connolly et al have noted:

Many advanced tumors produce excessive amounts of Transforming Growth Factor-β (TGF-β) which, in normal epithelial cells, is a potent growth inhibitor. However, in onco-genically

[9] https://www.ncbi.nlm.nih.gov/gene/7040

activated cells, the homeostatic action of TGF-β is often diverted along alternative pathways. Hence, TGF-β signaling elicits protective or tumor suppressive effects during the early growth-sensitive stages of tumorigenesis. However, later in tumor development when carcinoma cells become refractory to TGF-β-mediated growth inhibition, the tumor cell responds by stimulating pathways with tumor progressing effects.

At late stages of malignancy, tumor progression is driven by TGF-β overload. The tumor microenvironment is a target of TGF-β action that stimulates tumor progression via pro-tumorigenic effects on vascular, immune, and fibroblastic cells. Bone is one of the richest sources of TGF-β in the body and a common site for dissemination of breast cancer metastases. Osteoclastic degradation of bone matrix, which accompanies establishment and growth of metastases, triggers further release of bone-derived TGF-β. This leads to a vicious positive feedback of tumor progression, driven by ever increasing levels of TGF-β released from both the tumor and bone matrix.

It is for this reason, that pharmaceutical companies have developed therapeutic agents that block TGF-β signaling. Nonetheless, the choice of drug design and dosing strategy can affect the efficacy of TGF-β therapeutics. This review will describe pre-clinical and clinical data of four major classes of TGF-β inhibitor, namely i) ligand traps, ii) antisense oligonucleotides, iii) receptor kinase inhibitors and iv) peptide aptamers. Long term dosing strategies with TGF-β inhibitors may be ill-advised, since this class of drug has potentially highly pleiotropic activity, and development of drug resistance might potentiate tumor progression.

Current paradigms for the use of TGF-β inhibitors in oncology have therefore moved towards the use of combinatorial therapies and short-term dosing, with considerable promise for the clinic.

As Marin Acevedo et al note:

Transforming growth factor (TGF)-β is a cytokine that helps maintain tissue homeostasis by regulating cellular growth, differentiation, proliferation, and survival [50]. Although this pathway is able to control early-stage tumors by promoting cell cycle arrest and apoptosis, in advanced stages, it allows for tumor evasion by suppressing cytotoxic T cells and promotes cancer cell proliferation, invasion, and metastases, a functional switch known as the "TGF-β paradox".

Malignant cells achieve this switch through either the inactivation of their TGF-β receptors, or by selectively disabling the tumor-suppressive arm of this pathway, allowing cancer cells to use the TGF- β regulatory functions to their advantage by promoting immune tolerance. In fact, tumors that produce high levels of TGF-β can shield themselves from immune surveillance. Consistently, increased TGF-β expression by NSCLC, CRC, gastric, and prostate cancer has correlated with tumor progression and poor prognosis.

As Bassani et al note:

Suppressive cytokines are crucial orchestrators in shaping NK cell anergy and exhaustion in tumors. TGF-β is a major immunosuppressive cytokine present in the TME and it is detected at

high levels in different tumors. The inhibitory effects of TGF-β on NK cells are well documented and act mainly by downregulating the expression of NKG2D. TGF-β has also been shown to inhibit CD16-mediated human NK cell IFN-γ production and ADCC through SMAD3 [39].

5.1.3 KIR

KIR are receptors, transmembrane proteins. Abbas et al (9th Ed) note:

Many of the NK cell–activating receptors are called ***killer cell immunoglobulin (Ig)-like receptors (KIRs)*** *because they contain a structural domain named the immunoglobulin (Ig) fold, first identified in antibody (also known as Ig) molecules,*

Killer immunoglobulin–like receptors (KIR) are described by Vilches and Parham as follows:

KIR genes have evolved in primates to generate a diverse family of receptors with unique structures that enable them to recognize MHC-class I molecules with locus and allele-specificity. Their combinatorial expression creates a repertoire of NK cells that surveys the expression of almost every MHC molecule independently, thus antagonizing the spread of pathogens and tumors that subvert innate and adaptive defense by selectively downregulating certain MHC class I molecules. The genes encoding KIR that recognize classical MHC molecules have diversified rapidly in human and primates; this contrasts with conservation of immunoglobulin- and lectin-like receptors for nonclassical MHC molecules.

As a result of the variable KIR-gene content in the genome and the polymorphism of the HLA system, dissimilar numbers and qualities of KIR:HLA pairs function in different humans. This diversity likely contributes variability to the function of NK cells and T-lymphocytes by modulating innate and adaptive immune responses to specific challenges.

Beziat et note, KIR have a complex set of actions:

Human natural killer (NK) cells are functionally regulated by killer cell immunoglobulin like receptors (KIRs) and their interactions with HLA class I molecules. As KIR expression in a given NK cell is genetically hard-wired, we hypothesized that KIR repertoire perturbations reflect expansions of unique NK-cell subsets and may be used to trace adaptation of the NK-cell compartment to virus infections.

By determining the human "KIR-ome" at a single-cell level in more than 200 donors, we were able to analyze the magnitude of NK cell adaptation to virus infections in healthy individuals. Strikingly, infection with human cytomegalovirus (CMV), but not with other common herpesviruses, induced expansion and differentiation of KIR-expressing NK cells, visible as stable imprints in the repertoire.

Education by inhibitory KIRs promoted the clonal-like expansion of NK cells, causing a bias for self-specific inhibitory KIRs. Furthermore, our data revealed a unique contribution of activating KIRs (KIR2DS4, KIR2DS2, or KIR3DS1), in addition to NKG2C, in the expansion of human NK cells. These results provide new insight into the diversity of KIR repertoire and its adaptation to

virus infection, suggesting a role for both activating and inhibitory KIRs in immunity to CMV infection.

As Pittari et al note, the KIR fall into multiple classes:

The function of NK cells is governed by a set of germline- encoded activating or inhibitory receptors referred to as killer immunoglobulin-like receptors (KIRs).

The extracellular domain determines which HLA class I molecule NK cells recognize, whereas the intracytoplasmic domain transmits either an activating or an inhibitory signal.

KIRs are monomeric receptors with either 2 (KIR2D) or 3 (KIR3D) immunoglobulin-like domains, and are further subdivided into those with long (L) cytoplasmic tails (KIR2DL and KIR3DL) and short (S) cytoplasmic tails (KIR2DS and KIR3DS). Long-tail KIRs generate an inhibitory signal through the recruitment of the SH2-domain- containing tyrosine phosphatase 1 protein (SHP1).

Short-tail KIRs possess truncated portions that transduce activating signals via tyrosine phosphatase of DAP12 and other proteins.

The NK receptors are also a key element for potential immunotherapy. The KIR receptors are especially significant in this case.

5.1.4 PI3Kγ

Marin-Acevedo et al have described this in some detail as follows:

The expression of Phosphoinositide 3-kinase gamma (PI3Kγ) by macrophages controls a critical switch towards immune suppression in presence of inflammation and cancer. Additionally, PI3Kγ seems to play a role in angiogenesis by affecting the function of tumor-associated macrophages, major producers of VEGF[10]. Thus, similar to TGF-β, blocking this pathway exerts an indirect antitumor effect by modifying the microenvironment, improving the immunological function against malignant cells, and affecting the tumor vasculature. Unfortunately, as with other forms of immunotherapy, blocking PI3K enzymes has been associated with multiple autoimmune-like toxicities, and therefore the use of lower doses in conjunction with other forms of immunotherapy is often used.

5.1.5 CD47

CD47 is found in all hematopoietic cells as well as epithelial, endothelial, and fibroblast cells. It is associated with leukocyte adhesion, migration and activation and is a strong inhibitor to phagocytes.

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

5.2 STIMULATORY PATHWAYS

The stimulatory pathways are ones which enhance the action of the immune system and its functions. The cancer cells have means and methods to deactivate these.

5.2.1 OX40

We start with OX40. OX40 has certain stimulatory capabilities as NCBI notes[11]:

The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor has been shown to activate NF-kappaB through its interaction with adaptor proteins TRAF2 and TRAF5. Knockout studies in mice suggested that this receptor promotes the expression of apoptosis inhibitors BCL2 and BCL2l1/BCL2-XL, and thus suppresses apoptosis. The knockout studies also suggested the roles of this receptor in CD4+ T cell response, as well as in T cell-dependent B cell proliferation and differentiation.

Clinically, as Shtivelman et al note:

OX40 is not involved in effector T cell activation, but rather, promotes T cell survival and expansion. In a clinical study, ...patients received three infusions of the agonistic mouse anti-OX40 antibody within a week. The nature of the antibody precluded further treatments. Nine of 27 patients experienced minor tumor shrinkage, although none met RECIST (response evaluation criteria in solid tumors) criteria for objective responses

Now Marin Acevedo et al note:

OX40 (CD134) is a member of the TNF receptor super family, highly expressed by activated CD4, CD8 T cells, and Tregs, and in a lesser degree by neutrophils and NK cells. This molecule, along with its ligand, OX40L, plays a pivotal role in activation, potentiation, proliferation, and survival of T cells and modulation of NK cell function. Furthermore, this molecule inhibits the suppressive activity of Tregs by directly interfering with their function and proliferation, and indirectly antagonizing their inhibitory byproducts (e.g., TGFβ). Importantly, when tumor antigens are recognized by TILs, its expression of OX40 increases, and not surprisingly, the amount of OX40-expressing TILs correlates with improved prognosis in certain populations

5.2.2 GITR

First, NCBI describes this gene as[12]:

This gene encodes a member of the TNF-receptor superfamily. The encoded receptor has been shown to have increased expression upon T-cell activation, and it is thought to play a key role in dominant immunological self-tolerance maintained by CD25(+)CD4(+) regulatory T cells.

[11] https://www.ncbi.nlm.nih.gov/gene/7293

[12] https://www.ncbi.nlm.nih.gov/gene/8784

Knockout studies in mice also suggest the role of this receptor is in the regulation of CD3-driven T-cell activation and programmed cell death. Three alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported.

As Shtivelman et al note:

GITR is a costimulatory receptor expressed after T cell activation that enhances T cell function and survival. Importantly, GITR also negatively affects regulatory T cells (Tregs), and treatment with GITR agonistic antibody destabilizes intra-tumor Tregs allowing for more efficient cytolysis by CD8+ T cells [246]. A trial with anti-GITR antibody TRX-518 is ongoing in melanoma patients.

5.2.3 ICOS

We begin by the NCBI definition of ICOS as follows[13]:

The protein encoded by this gene belongs to the CD28 and CTLA-4 cell-surface receptor family. It forms homodimers and plays an important role in cell-cell signaling, immune responses, and regulation of cell proliferation.

Now Marin Acevedo et al note in further detail:

Inducible co-stimulator (ICOS), a specific T cell costimulatory molecule of the CD28/CTLA-4 family mainly expressed by CD4 T cells, is a co-stimulator of proliferation and cytokine production by these cells. Its levels are upregulated in activated T lymphocytes, especially after the use of anti-CTLA4 therapies, and its expression is considered a biomarker to indicate that anti-CTLA4 agents are binding its target.

Increased ICOS expression on circulating T cells after ipilimumab administration has been associated with improved clinical outcomes. Interestingly, ICOS appears to be a less potent pathway compared to other forms of immunotherapy mainly because of a predominant CD4 expression. However, its use with other approaches, particularly CTLA4 blockade, can lead to a potent synergistic effect as a result of an increase in the expression of ICOS after anti-CTLA4 therapy

5.2.4 CD40

We begin with NCBI which states[14]:

This gene is a member of the TNF-receptor superfamily. The encoded protein is a receptor on antigen-presenting cells of the immune system and is essential for mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class

[13] https://www.ncbi.nlm.nih.gov/gene/29851

[14] https://www.ncbi.nlm.nih.gov/gene/958

switching, memory B cell development, and germinal center formation. AT-hook transcription factor AKNA is reported to coordinately regulate the expression of this receptor and its ligand, which may be important for homotypic cell interactions.

Adaptor protein TNFR2 interacts with this receptor and serves as a mediator of the signal transduction. The interaction of this receptor and its ligand is found to be necessary for amyloid-beta-induced microglial activation, and thus is thought to be an early event in Alzheimer disease pathogenesis. Mutations affecting this gene are the cause of autosomal recessive hyper-IgM immunodeficiency type 3 (HIGM3). Multiple alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported.

As Shtivelman et al have noted regarding CD40:

CD40. Unlike the costimulatory targets above, CD40 is expressed on APCs, while its ligand is expressed on T cells. Binding of the two acts as a powerful enhancer of APCs' ability to present antigens and activate T cells against foreign targets. A large number of cancer patients received infusions of agonistic antibody CP870,893 and some responses were observed [247]. A surprising finding was that treatments did not increase numbers of TILs in the tumors. In a mouse model, antibody treatments induced an influx of macrophages into tumors, presumably with enhanced cytotoxic activities.

5.3 OTHER PATHWAYS

There are many other possible controlling pathways as well. We follow Marin-Acevedo et al in discussing some of them.

5.3.1 IDO

NCBI indicates two types of IDO, IDO1 and IDO2. For IDO1 NCBI notes[15]:

This gene encodes indoleamine 2,3-dioxygenase (IDO) - a heme enzyme that catalyzes the first and rate-limiting step in tryptophan catabolism to N-formyl-kynurenine. This enzyme acts on multiple tryptophan substrates including D-tryptophan, L-tryptophan, 5-hydroxy-tryptophan, tryptamine, and serotonin. This enzyme is thought to play a role in a variety of pathophysiological processes such as antimicrobial and antitumor defense, neuropathology, immunoregulation, and antioxidant activity. Through its expression in dendritic cells, monocytes, and macrophages this enzyme modulates T-cell behavior by its peri-cellular catabolization of the essential amino acid tryptophan.

For IDO2 NCBI notes[16]:

[15] https://www.ncbi.nlm.nih.gov/gene/3620

[16] https://www.ncbi.nlm.nih.gov/gene/169355

Along with the enzymes encoded by the INDO (MIM 147435) and TDO2 (MIM 191070) genes, the enzyme encoded by the INDOL1 gene metabolizes tryptophan in the kynurenine pathway.

Now Abbas et al (8th) note:

Immune responses to the fetus may be regulated by local concentrations of tryptophan and its metabolites in the decidua, which inhibit T cell responses. The enzyme indoleamine 2,3-dioxygenase (IDO) catabolizes tryptophan, generating a byproduct, kynurenine. Tryptophan is required for proliferating cells, including lymphocytes, and kynurenine is toxic to these cells. These observations led to the hypothesis that T cell responses to the fetus are normally blocked because decidual tryptophan levels are kept low or the levels of toxic metabolites produced by IDO are high.

From Prendergast we have further detail regarding this target:

Immune escape is a critical gateway to malignancy. The emergence of this fundamental trait of cancer represents the defeat of immune surveillance, a potent, multi-armed and essential mode of cancer suppression that may influence the ultimate clinical impact of an early stage tumor. Indeed, immune escape may be a central modifier of clinical outcomes, by affecting tumor dormancy versus progression, licensing invasion and metastasis and impacting therapeutic esponse. Although relatively little studied until recently, immune suppression and escape in tumors are now hot areas with clinical translation of several new therapeutic agents already under way. The interconnections between signaling pathways that control immune escape and those that control proliferation, senescence, apoptosis, metabolic alterations, angiogenesis, invasion and metastasis remain virtually unexplored, offering rich new areas for investigation.

Here, an overview of this area is provided with a focus on the tryptophan catabolic enzyme indoleamine 2,3-dioxygenase (IDO) and ***its recently discovered relative IDO2 that are implicated in suppressing T-cell immunity in normal and pathological settings including cancer.***

Note that it is IDO2 that is alleged to do the suppressing, not the IDO1. They continue:

Emerging evidence suggests that during cancer progression activation of the IDO pathway might act as a preferred nodal modifier pathway for immune escape, for example analogous to the PI3K pathway for survival or the VEGF pathway for angiogenesis.

Small molecule inhibitors of IDO and IDO2 heighten chemotherapeutic efficacy in mouse models of cancer in a nontoxic fashion and an initial lead compound entered phase I clinical trials in late 2007. New modalities in this area offer promising ways to broaden the combinatorial attack on advanced cancers, where immune escape mechanisms likely provide pivotal support.

5.3.2 TLR

Toll Like Receptors are powerful elements in the innate immune system. The Toll Like Receptors, "toll" means weird or strange in German, play a significant role in the innate system. As Travis notes:

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

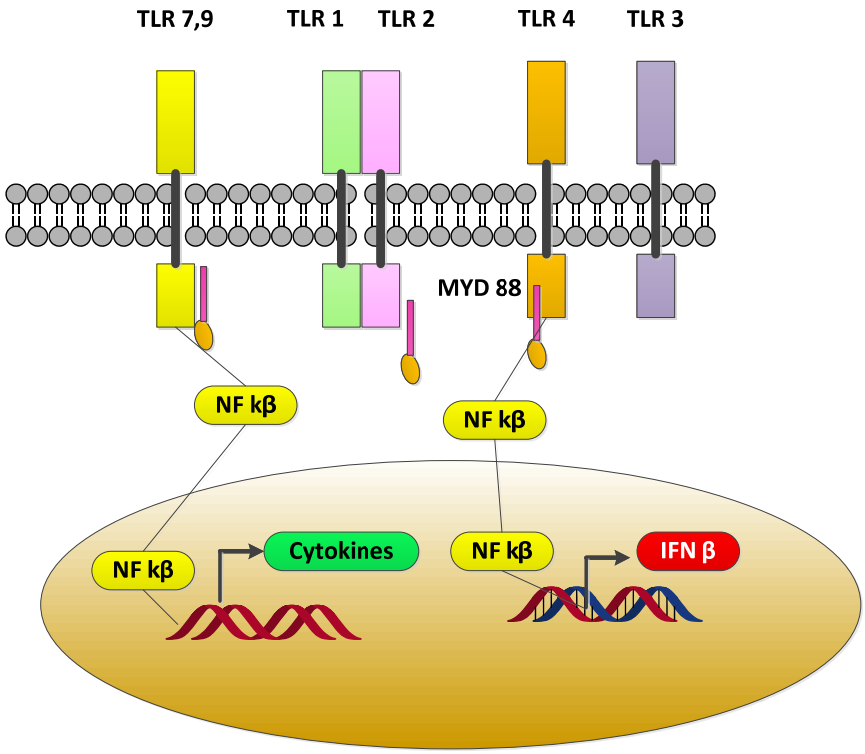
Takeda and Ashira note:

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

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

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

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



We will see more from these TLR as we proceed.

5.3.3 IL-2R

There are several variants of IL-2R, namely IL-2RA[17], IL-2RB[18] and IL-2RG[19] to name a few. Marin Acevedo et al note:

IL-2 mediates its immune-enhancing effect through either a low-affinity dimeric and/or a high-affinity trimeric IL-2 receptor (IL-2R). The dimeric IL-2R consists of CD122 (also known as IL-2Rβ) and CD132 (also known as Yc), whereas the trimeric IL-2R comprises an additional component, the CD25 (also known as IL-2Rα) which increases the affinity for its ligand

5.3.4 Arginase Inhibitor

Marin Acevedo et al note:

[17] https://www.ncbi.nlm.nih.gov/gene/3559

[18] https://www.ncbi.nlm.nih.gov/gene/3560

[19] https://www.ncbi.nlm.nih.gov/gene/3561

Arginine is an important amino acid for T cell activation and proliferation. High levels of arginase are produced by malignant cells and MDSCs leading to depletion of arginine and a subsequent immunosuppressive tumor microenvironment. The use of arginase inhibitors could allow overcoming the immunosuppressive effects of the tumor microenvironment and achieve a better antitumor control with the use of other immune checkpoint inhibitors or radiation therapy. Furthermore, the blockade of arginase may also have direct antitumor effects by decreasing the availability of substances that favor tumor growth. Finally, given a higher expression of arginine among the tumor microenvironment than that in plasma, the use of these molecules could be associated with a more specific and less toxic effect than other forms of immunotherapy.

5.3.5 Oncolytic Peptides

In simple terms, Stiberg states[20]:

Immunotherapy is a type of cancer treatment that involves stimulating the body's immune system to recognise and destroy cancer cells. So-called oncolytic peptides, which are a chain of amino acids, form the basis for this new type of immunotherapy against cancer. The injection of these peptides into a tumour releases signals that stimulate the immune system. Moreover, the process activates a large amount of the patient's own antigens, which in turn strengthens the fight against the cancer tumour.

As Eksteen et al note:

Oncolytic peptides represent a promising new strategy within the field of cancer immunotherapy. Here we describe the systematic design and evaluation of short antilymphoma peptides within this paradigm. The peptides were tested in vitro and in vivo to identify a lead compound for further evaluation as novel oncolytic immunotherapeutic. In vitro tests revealed peptides with high activity against several lymphoma types and low cytotoxicity toward normal cells. Treated lymphoma cells exhibited a reduced mitochondrial membrane potential that resulted in an irreversible disintegration of their plasma membranes. No caspase activation or ultrastructural features of apoptotic cell death were observed.

One of these peptides, was shown to induce complete tumor regression and protective immunity following intralesional treatment of murine A20 B-lymphomas. Due to its selectivity for lymphoma cells and its ability to induce tumor-specific immune responses, has the potential to be used in intralesional treatment of accessible lymphoma tumors.

As Gaspar et al note[21]:

Antimicrobial peptides (AMPs) are part of the innate immune defense mechanism of many organisms. Although AMPs have been essentially studied and developed as potential alternatives for fighting infectious diseases, their use as anticancer peptides (ACPs) in cancer therapy either

[20] https://norut.no/en/news/nok-10-million-liver-cancer-research

[21] The authors note that these are also called oncolytic peptides

alone or in combination with other conventional drugs has been regarded as a therapeutic strategy to explore.

As human cancer remains a cause of high morbidity and mortality worldwide, an urgent need of new, selective, and more efficient drugs is evident. Even though ACPs are expected to be selective toward tumor cells without impairing the normal body physiological functions, the development of a selective ACP has been a challenge. It is not yet possible to predict antitumor activity based on ACPs structures. ACPs are unique molecules when compared to the actual chemotherapeutic arsenal available for cancer treatment and display a variety of modes of action which in some types of cancer seem to co-exist.

Regardless the debate surrounding the definition of structure-activity relationships for ACPs, great effort has been invested in ACP design and the challenge of improving effective killing of tumor cells remains. As detailed studies on ACPs mechanisms of action are crucial for optimizing drug development, in this review we provide an overview of the literature concerning peptides' structure, modes of action, selectivity, and efficacy and also summarize some of the many ACPs studied and/or developed for targeting different solid and hematologic malignancies with special emphasis on the first group.

5.3.6 IL-10

We begin with NCBI which notes[22]:

The protein encoded by this gene is a cytokine produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines, MHC class II Ags, and costimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. ***This cytokine can block NF-kappa B activity, and is involved in the regulation of the JAK-STAT signaling pathway.*** *Knockout studies in mice suggested the function of this cytokine as an essential immunoregulator in the intestinal tract. Mutations in this gene are associated with an increased susceptibility to HIV-1 infection and rheumatoid arthritis*

Now from the work of Marin Acevedo et al they note:

IL-10 inhibits secretion of proinflammatory cytokines (e.g., IFNγ, TNFα, IL-1β, IL-6) and also inhibits the expression of MHC molecules and costimulatory molecules at several levels, leading to inhibition of T cell function. Recently, IL-10 was also found to play some antitumor role by inducing the activation and proliferation of CD8. CD8 cells expressing IL-10 has been associated with a favorable prognosis in patients with lung cancer.

However, similar to other interleukins like IL-2, its effects are pleotropic and this raises concern for potential systemic toxicity. Other unresolved issues similar to IL-2 therapy include determining the patient population that could benefit the most from this form of therapy and the

[22] https://www.ncbi.nlm.nih.gov/gene/3586

most appropriate therapeutic combinations. In this regard, both PD-1 and IL-10 receptors are upregulated in TILs and therefore the combined use of these molecules is reasonable.

6 OBSERVATIONS

Having examined a multiplicity of existing and putative immunotherapeutic targets for the control of cancers we now present several observations which may act as a basis for extension. Some of the observations here are more detailed upon what we discussed earlier and others are anticipatory of possible extensions.

The first two, the tumor micro environment and tumor associated macrophages are we believe critical factors to be considered while examining immunotherapy. They build upon one another creating a powerful self-sustaining stronghold for cancer clusters.

6.1 TUMOR MICRO-ENVIRONMENT (TME)

We have mentioned the TME previously and in some detail in the introduction. However, it is important to understand that attempting to use the immune system one must deal with the totality of a tumors defenses and key amongst them is the TME. As Murgaski et al have noted:

It is becoming increasingly apparent that our immune system is capable of fighting cancer. Understanding the interplay between our immune system and cancer has led to the development of new treatments that can prolong survival in once-thought terminal patients. The success that immune checkpoint inhibitors (ICIs) have had in the clinic has sparked renewed interest and investment in the tumour immunology field. However, durable responses to immunotherapy are only seen in a minority of patients.

A common trait among many treatment responsive patients is a high neoantigen load; a characteristic which often correlates with a strong adaptive immune response against the tumour. This response is required for the ICIs to release the brakes that the tumour places on the immune system.

On the other hand, patients who do present a high neoantigen load may not respond to ICIs due to an immunosuppressive tumour microenvironment (TME). In these patients, anti-tumour immune responses are shut down by immunosuppressive cells such as tumour-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs). Interactions between these suppressive cells and effector T cells can lead to T-cell exhaustion, a state of T-cell dysfunction seen during chronic inflammation.

While ICIs can rescue some T cells from these interactions, suppression of the TME might still be too strong for T cells to fully overcome this obstacle, resulting in continued tumour progression. Therefore, it is imperative to improve the adaptive immune response against the tumour while simultaneously redirecting the TME toward a more immuno-permissive state. In this light, harnessing the potential of dendritic cells (DCs) that reside within tumours is one avenue of research that could yield positive clinical results for cancer patients in the near future.

It is well established that DCs have the ability to link both the innate and adaptive immune systems and to initiate immune responses. In the age of immunotherapy, this capacity to generate

adaptive immune responses is considered to be imperative. DCs can activate T-cell responses… with great efficacy due to their high expression of co-stimulatory molecules and specific T-cell adhesion molecules. However, DCs are also capable of shutting down immune responses by expressing high levels of co-inhibitory molecules. Therefore, understanding and exploiting mechanisms relating to the function of tumour-associated DCs (TADCs) can lead to the development of powerful tools to fight cancer.

6.2 TUMOR ASSOCIATED MACROPHAGES

Macrophages search out and target cells to be cleaned but by the immune system, most of the time. However, the tumor associated macrophages can act in a pro tumor manner actually enhancing tumor growth and activating metastatic behavior. As Huang et al note:

Tumor associated macrophages (TAMs) play an important role in tumorigenesis and progression. TAMs generate an inflammatory environment to trigger or facilitate tumor initiation, promote tumor cell invasion and metastasis, stimulate angiogenesis and suppress antitumor immunity. High density of TAMs was correlated with the poor prognosis of a wide range of tumors such as lung, hepatocellular, colorectal, breast, prostate, ovarian and thyroid cancers.

TAMs produced growth factors (e.g. VEGF, EGF, HGF and bFGF) and chemokines (e.g. CXCL12 and IL8) to mediate their oncogenesis function. On the other hand, cancer cells recruit TAMs by releasing colony stimulating factor (CSF1), granulocyte–monocyte (GM-CSF), transforming growth factor (TGF) or chemokines (e.g. CCL2)

In several thyroid excisions one can see the follicular and/or papillary cells but at the same time if one looks there may be large collections of macrophages. If that were to be the case then one may expect that the lesion has metastasized.

Noy and Pollard have noted:

Macrophages in the Primary Tumor: Cancer Initiation Tumors acquire mutations in oncogenes or tumor-suppressor genes that permit them to progress to malignancy. Although most cancer research has focused upon these changes and most therapeutics are directed against these tumor cells, it is now apparent that the nonmalignant cells in the microenvironment evolve along with the tumor and provide essential support for their malignant phenotype.

In fact both the systemic and local environment play a tumor-initiating role through the generation of a persistent inflammatory responses to a variety of stimuli. For example, obesity is associated with increased risk of many but not all cancers and is characterized by an enhanced systemic inflammatory response and locally, for example in the breast, to an increased number of inflammatory crown-like structures consisting of macrophage and adipocytes whose number strongly correlates with breast cancer risk.

Similarly persistent inflammation referred to as ‘‘smoldering inflammation’’ caused by chronic infection with viruses such as Hepatitis B virus in liver, bacteria like Helicobacter pylori in the

stomach, or due to continuous exposure to irritants such as asbestos in the lung is casually associated with cancer initiation.

Furthermore, inflammatory conditions such as Crohn's disease dramatically increase the risk of colorectal cancer. Inflammation always has a substantial macrophage involvement through their production of molecules such as interleukin-6 (IL-6), tumor necrosis factor-a (TNF-a), and interferon-g (IFN-g).

To support this correlative data between macrophage-mediated inflammation and cancer induction, … found that genetic ablation of the anti-inflammatory transcription factor Stat3 in macrophages results in a chronic inflammatory response in the colon that is sufficient to induce invasive adenocarcinoma. In addition, loss of the anti-inflammatory cytokine IL-10 that acts through STAT3 enhances carcinogen-induced tumorigenesis in the intestine.

Mechanistically, this inflammation can cause tumor initiation by creating a mutagenic microenvironment either directly through free radical generation or indirectly via alterations in the microbiome and barrier functions that allow access of genotoxic bacteria to the epithelial cells.

In fact, there are also metastasis associated macrophages, MAMs, which are a special class of TAM, in that they assist in the metastatic process. The TAMs actually suppress and block T cell action as well as NK and NKT cell actions. The TAMs create a protective buffer for the growth of the tumor and in a sense add an additional element to the tumor micro environment. In a strange sense it is the immune system itself which assists in the growth of the malignancy. The authors continue:

In addition to these MHC molecules, macrophages express the ligands of the inhibitory receptors programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4). These inhibitory ligands are normally upregulated in activated immune effector cells such as T cells, B cells, and NK T cells as part of a safety mechanism that controls the intensity of the immune response and as part of inflammation resolution. Activation of PD-1 and CTLA-4 by their ligands (PD-L1, PD-L2, and B7-1 [D80], B7-1 [CD86], respectively) directly inhibits TCR and BCR signaling.

This activation also inhibits T cell cytotoxic function, regulates their cell cycle, and inhibits their activation as CTLA4 competes with CD28 (costimulatory) binding. PD-L1 and PD-L2 are differentially expressed, with PD-L1 constitutively expressed by immune cells including T cells, B cells, macrophages, DCs, nonhematopoietic cells, and cancer cells.

In contrast, PD-L2 expression is limited to antigen- presenting cells (APCs). Its expression is induced in monocytes and macrophages by CSF1, IL-4, and INF-g. Both PD-L1 and L2 are regulated in TAMs and myeloid-derived suppressor cells.

Recently, …showed that MDSCs and TAMs in hypoxic tumor regions upregulate the expression of PD-L1 as a consequence of HIF-1a signaling (Noman et al., 2014). Hypoxia acting via hypoxia inducible factor 1- a (HIF-1a) also induces T cell suppression by TAMS although the mechanism is unknow. It has also been shown that monocytes from blood of glioblastoma

patients express higher amounts of PD-L1 compared to healthy donors and that glioblastoma-cell-conditioned medium can upregulate PD-L1 expression in monocytes from healthy donors.

Similarly, monocytes from patients with hepatocellular carcinoma express PD-L1 that contributes to human tumor xenograft growth in vivo, while the blocking of PD-L1 reverses this effect.

Thus, in a strange way the action of the macrophages sets up the PD-1 type of blockade we then try to work around. Perhaps as some author suggest we should also target the TAMs and MAMs.

6.3 SUMMARY OF OPTIONS

The following is a summary of the options based upon the Marin-Acevedo paper:

Class	Applications
Tumor-directed monoclonal antibodies	BiTE DART
Antibody drug conjugates	
CAR-T Cells	T4 immunotherapy Anti-CD19 CAR T cells (CART-19) Anti-GPC3 CAR T cells Anti-CD133 CAR T cells (CART-133) Anti-BCMA CAR T cells (bb2121) Anti-CD138 CAR T cells Anti-immunoglobulin kappa light chain CAR T cells Anti-CD30 CAR T cells Anti-IL13 CAR T cells
T cell receptor (TCR) gene-modified T cell therapy	Anti-NY-ESO-1 TCR T cells (NY-ESO-1c259t) Anti-E6 TCR T cells Anti-MAGE A10 TCR T cells
Tumor-infiltrating T cell therapy	
Oncolytic viruses	
Vaccines	Tumor cell vaccines Genetic vaccines Dendritic cell vaccines Protein/peptide-based vaccines In situ vaccines Neoantigen vaccines
Other approaches in immunotherapy	Targeting myeloid-derived suppressor cells Targeting tumor microenvironment Cytokine gene therapy Oncolytic peptides

As can be noted above, there is now an explosion of options that facilitates the use of the immune system to effect cancer mitigation.

6.4 OTHER PATHWAYS

We present a summary of the specific targets and their related APC or tumor element, corresponding T cell element and their inhibitory or stimulatory effects.

APC or Tumor	*T Cell*	*Inhibitory Pathway*	*Stimulatory Pathway*
A2aR	A2aR	X	
VISTA		X	
B7-H3		X	
PDL1/PDL2	PD1	X	
CD80/CD86	CTLA-4	X	
galectin-9	TIM-3	X	
OX40L	OX40		X
CD40	CD40L		X
B7RP	ICOS		X
CD70	CD27		X
HVEM	BTLA	X	
MHC I/II	LAG-3 TCR KIR	X	
GITRL	GITR		X
4-1 BBL	4-1 BB		X
CD155/CD112	CD226/TIGIT	X	X

6.5 Some Current Options

We now examine a set of the possible options available. From Manson and Houot who state:

The complex relationship between the immune system and cancer development has been the subject of investigation for decades. In recent years, crucial advances have been made in this field. This progress, combined with technological advances, has led to the development of novel immunotherapies which have demonstrated remarkable efficacy for the treatment of cancer. In lymphoid malignancies, three of these new immunotherapies appear to be particularly promising:

immune checkpoint inhibitors (CPI),

Tcell engager antibodies (TCE) and

chimeric antigen receptor (CAR)-T cells.

Each of these approaches has its own advantages and inconveniences (Table 1). Some of these immunotherapies have already been granted approval by the Food and Drug Administration (FDA) for hematologic malignancies [anti-PD1 antibodies (Abs) in Hodgkin lymphoma (HL), TCE and CAR-T cells in B-cell acute lymphoblastic leukemia (B-ALL)]. In the future, these approvals are likely to be extended to other malignancies, including HL and non-Hodgkin lymphoma (NHL). In this review, we analyze the most recent clinical data regarding these different immunotherapies in patients with lymphoma.

From Manson and Houot we have the following table which summarizes some of these approaches:

	Checkpoint Inhibitors	T Cell Engager Ab	CAR T Cells
Type of therapy	Antibody	Antibody	Adoptive cell therapy
Mechanism of action	Block inhibitory signals on T cells	Recruit and activate T cells at the tumor site	Genetically modified T cells recognize and kill tumor cells
Requirement for tumor Ag identification	No	Yes	Yes
Specificity against tumor cells	Polyclonal	Monoclonal	Monoclonal
Nature of Ag targeted	Intracellular and surface	Surface	Surface
HLA-restricted recognition of Ag Yes	No	No	
Long-lasting protection	Yes	No	Yes
Off-the-shelf	Yes	Yes	No
Administration	Sequential	Continuous	Single
Half-life	Weeks	Hours	Months/Years
Personalized therapy	0	+	+++
Main toxicities	Immune-related adverse events	Neurotoxicity	Cytokine release syndrome Neurotoxicity
FDA-approved for cancer	Anti-CTLA-4: ipilimumab: Anti-PD-1: nivolumab, pembrolizumab Anti-PDL-1: atezolizumab, avelumab, durvalumab	Anti-CD3/CD19 blinatumomab	CD19 CAR-T: KTE-C19, CTL-019

6.6 UNINTENDED CONSEQUENCES

Various "storms" of cytokines and others putatively harmful immune cell products have been known to have taken their toll, well ahead of the malignancy. As Tisoncik et al note:

Inflammation associated with a cytokine storm begins at a local site and spreads throughout the body via the systemic circulation. Rubor (redness), tumor (swelling or edema), calor (heat),

dolor (pain), and "functio laesa" (loss of function) are the hallmarks of acute inflammation. When localized in skin or other tissue, these responses increase blood flow, enable vascular leukocytes and plasma proteins to reach extravascular sites of injury, increase local temperatures (which is advantageous for host defense against bacterial infections), and generate pain, thereby warning the host of the local responses.

These responses often occur at the expense of local organ function, particularly when tissue edema causes a rise in extravascular pressures and a reduction in tissue perfusion. Compensatory repair processes are initiated soon after inflammation begins, and in many cases the repair process completely restores tissue and organ function. When severe inflammation or the primary etiological agent triggering inflammation damages local tissue structures, healing occurs with fibrosis, which can result in persistent organ dysfunction.

The cytokines involved include: interferons, interleukins, chemokines, CSFs, TNFs. A massive release of some cocktail of these can then play havoc on a systemic basis. We summarize these below:

Type	Actions
Interferons	Regulation of innate immunity, activation of antiviral properties, antiproliferative effects
Interleukins	Growth and differentiation of leukocytes; many are proinflammatory
Chemokines	Control of chemotaxis, leukocyte recruitment; many are proinflammatory
Colony Stimulating Factors	Stimulation of hematopoietic progenitor cell proliferation and differentiation
Tumor necrosis factors	Proinflammatory, activates cytotoxic T lymphocytes

Tokuyasu and Huang have noted:

Cancer immunotherapies can in principle have much milder side effects compared to radiotherapy and chemotherapy. In practice, they are associated with their own spectrum of adverse events. In particular, cytokine release syndrome ("cytokine storm") can lead to organ failure and death. Both treatment efficacy and adverse events are associated with proliferative and persistent cellular responses, which can vary significantly between individuals, thus requiring careful monitoring. Adverse events associated with neoantigen vaccines appear to be relatively mild, compared to adoptive cell transfer, checkpoint blockade, and tumor-associated antigen (TAA) vaccine therapies.

6.7 ADAPTIVE VS INNATE

Much of the focus is on T cells and the T cell activation that results. That is we see the use of the adaptive system, bypassing the classic B cell activation and resulting chain of events. However, the innate system, to some degree the complement portion, presents another multiplicity of

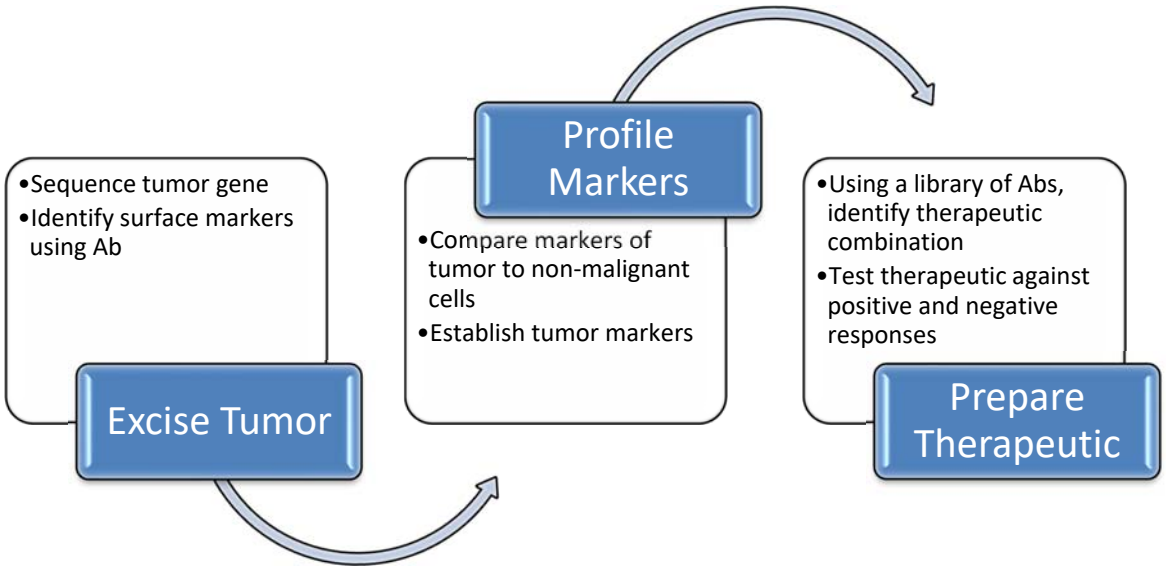
options. The innate system is a sledgehammer approach but in certain ways this can be more effective. One should expect that as we can mitigate the sledgehammer effects that innate approaches will multiply.

6.8 TOOLS

The key to many of these new approaches, besides just basic knowledge, is the availability of tools. These are new ways to identify and measure what we are seeking.

6.9 INDIVIDUALIZED TARGETING

The key question is; how can we put these new insights to clinical use? We believe that a personalized medicine targeting these cells is possible in a near production mode. We demonstrate such a paradigm below.



The problem, however, is that we need production level tools. Research level tools are still cumbersome and of limited interest. Moreover, we still at times struggle with identifying cancerous cells[23].

[23] https://www.researchgate.net/publication/334947163_What_is_Meant_by_Cancer

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