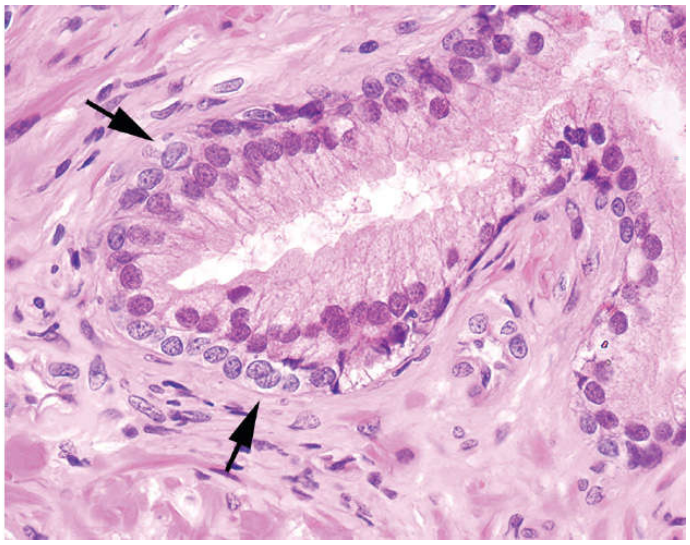

PROSTATE CANCER: CAR-NK OR AB TGL 196

ABSTRACT

We examine prostate cancer cells for a stem cell target and on that cell specific surface markers that are targetable. We then consider two general mechanisms to attack those malignant cells, CAR methods focusing on NK cells and antibody methods using an amalgam of surface targets.

TERRENCE MCGARTY

FEBRUARY 2023

Notice

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

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Our approach herein is to take elements of what is recent in the literature focused on a specific topic and attempt to develop a tapestry image of these connectable elements. We do not provide any new or fundamental results but merely attempt to assemble elements in a systematic manner.

The Author would appreciate any communications relating to these documents and these should be sent to:

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Contents

1 INTRODUCTION

Cancer therapeutics are a continuing expanding base. Initially classic chemotherapy was in many ways a carpet bombing of all cells. The chemotherapeutics just attacked any cells progression with total disregard to what cell was healthy and what cell was not. The results were often significant co-morbidity. The recent explosion of monoclonal antibodies has added a more nuanced element in the armamentarium. Namely having gathered more information on the nature of the cells in the specific malignancy, such as HER2+ breast cancer, MAb can be generated to attack those cells.

Again there are still other cells that get affected resulting in co-morbidity. Thus there is the swinging interaction between understanding more about the cancer cell specificity and more accurate targeting of a therapeutic. The same principle holds true in immune system responses. Thus CAR-T cells targeting the surface marker, CD19, work well for many patients but may cause cytokine storms or GVHD in others. The targeting is still a bit of an overshoot. In this report we examine improved specificity of targeting, how it may be achieved, and how it may be used in CAR like immune attacks of polyclonal antibodies, PAb. The specific malignancy examine will be prostate cancer.

As King has noted:

Some well-known characteristics of prostate tumours seem to make them difficult targets for immunotherapy.

One is that prostate cancers tend to mutate less than other cancers, and therefore present fewer targets for immune cells. "There's just not as much fodder for T cells to recognize," says Charles Drake, vice-president of immuno-oncology at Janssen Pharmaceutical and an oncologist at Columbia University Medical Centre in New York City.

Another is that the prostate has an unusually dialled-down immune environment. "There are not a lot of immune cells in the prostate when you do biopsies, and most of those there are not activated," says James Gulley, an immuno-oncologist at the US National Institutes of Health (NIH) in Bethesda, Maryland. Researchers using genetic sequencing to look for immune cells in the prostate will often come away empty-handed. "If you analysed the entire tumour, you would miss the signal from T cells because there are so few," says Julie Graff, a prostate oncologist at Oregon Health & Science University (OHSU) in Portland. This poses a problem for checkpoint inhibitors. If there is a shortage of T cells to begin with, simply switching on those that are there is unlikely to do much good. …

***Drake thinks that immunotherapy should be given before hormone therapy**. Once patients have their testosterone levels lowered in this way, T cells make their way to the prostate gland and inflammatory cytokines are released. But what comes next is a wave of cells that suppress the immune response, says Drake, so delivering immunotherapy ahead of that could be advantageous. It could potentially even remove the need for hormone therapy altogether and avert its side effects … Ultimately, prostate-cancer treatment is playing catch-up.*

*"**Prostate cancer is about 15 years behind some of the other cancers," says Haas. She points out that it wasn't until 1996 that chemotherapy was effective for prostate cancer. Questions about the tumour microenvironment and how best to target immunotherapy are well on their way to being answered for many other cancers, but still hold much mystery in prostate cancer.** "We haven't quite cracked it," says Gulley, "but there is optimism that there will be a path forward.*

Prostate cancer, PCa, is the most significant for men across most countries[1]. It generally occurs in an older age group and in most cases is a slowly progressing lesion. Treatment of PCa has been problematic, surgery and radiation being the dominant options. Surgery has a positive side and a negative. On the positive one gets to biopsy the cells and determine a better prognosis. On the negative side there may be nerve damage and other local damage that may result in patient disabilities.

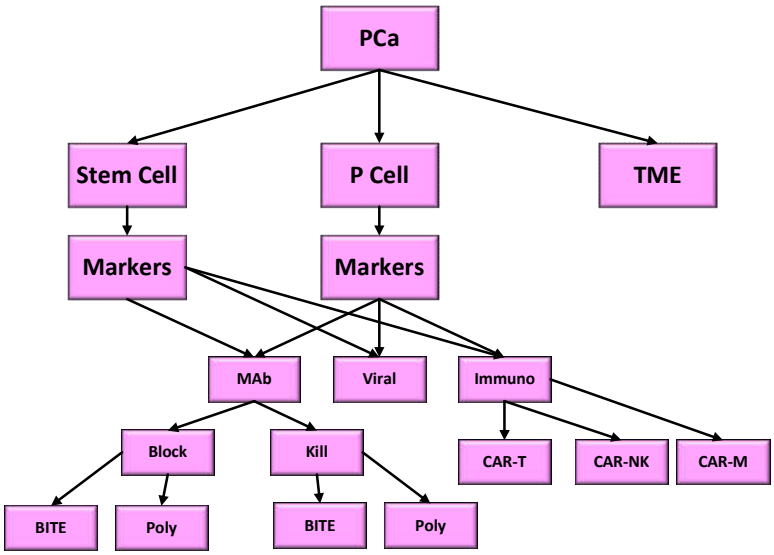
There has been some progress in the use of therapeutics but unlike some other cancers the targeting has been less successful. We examine herein the various ways in which PCa may be addressed via a therapeutic means. Key to these approaches is an ability to identify the malignant cells and to accurately target them and no others.

1.1 OPTIONS

The chart below outlines some options. We will examine the different path options that are currently available or under development. Our intent herein is to explore these options[2]. It must be noted, however, that much of this is currently speculative, some in clinical trials, and a limited amount in actual clinical application. The challenge in PCa is that it most likely be the cancer of highest incidence and prevalence in males, namely and equivalently those individuals with the Y chromosome. Thus any approach to its mitigation could have far reaching positive effects.

[1] See https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach (2012) This was a review from a Systems Perspective of PCa at the time. This document extends significantly many issues developed in the interim. Also a full list of reports is available in https://www.researchgate.net/profile/Terrence-Mcgarty/research

[2] See Wendel et al for a recent overview as well



Let us consider the first level. Namely we look at attacking one or more of the following:

1. Stem Cell: The stem cell is a construct that posits a cell which is the master controller of the other cells, namely the proliferative sells. The stem cell, if it exists, can be targeted and it so then arguably the proliferation ceases.

2. Proliferative Cell (PC): These are the cells that are offshoots of the Stem Cell. They are the bulk of the malignancy.

3. Tumor micro-environment (TME): Oftentimes we have a TME which protects the stem and proliferative cells. This may include a variety of cells such as macrophages and fibroblasts. It may be essential to attack this protective environment as well.

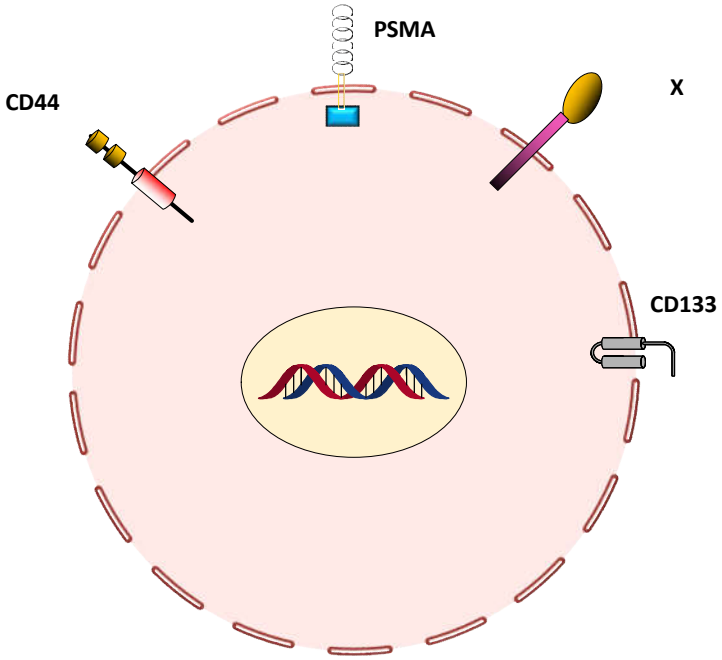
Now one can address each of these by first understanding what identifies a malignant cell, namely a surface marker or better yet an agglomeration of specific surface markers. Then we can attack these specific cells by one of several means, three of which we discuss here; (i) immune attack with T cells, NK cells and the like, (ii) polyclonal antibodies attaching to a set of markers and delivering a chemotherapeutic load to destroy the cell, (iii) a viral attack to cell surface targets to liquidate the cancer cell.

We consider the first two in this report. Namely immune techniques and antibody techniques. We defer viral techniques to a later analysis.

1.2 TARGETS

The issues we examine herein are simply, given a cell with a known set of surface targets, can we develop a therapeutic to attack that cell and neutralize it? We may assume that we would have to assess the markers on a patient by patient basis and on a cell by cell basis. Then knowing

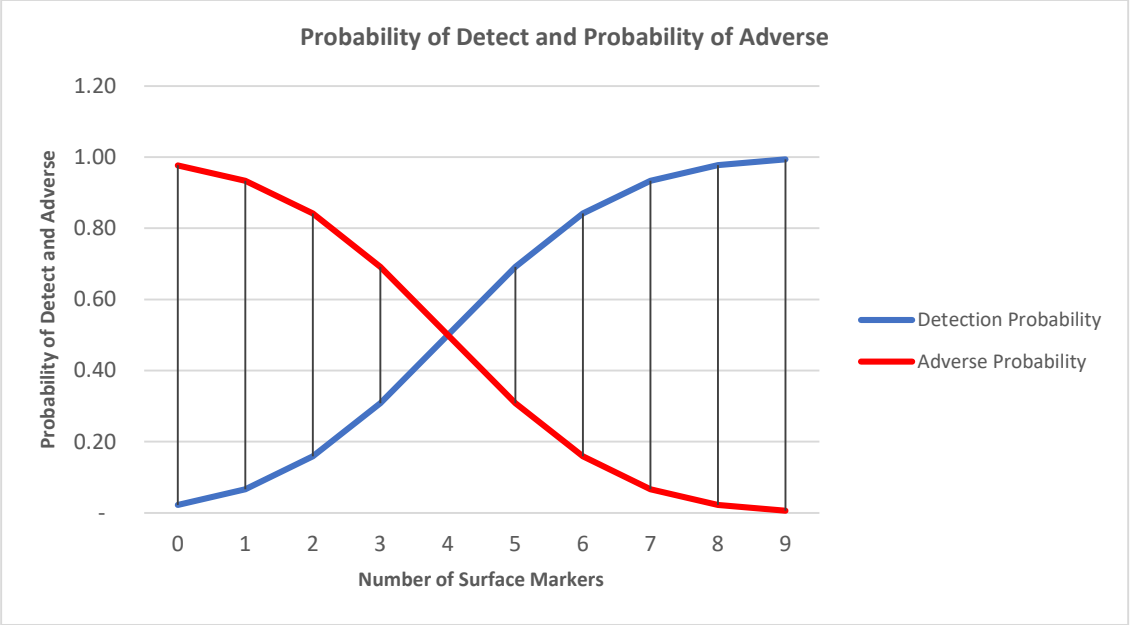
that we can then address a neutralization strategy based upon one or more of the options discussed previously.



In essence we are creating a truly personalized therapeutic. Specifically it is both patient dependent as well as disease dependent.

1.3 PARADIGM

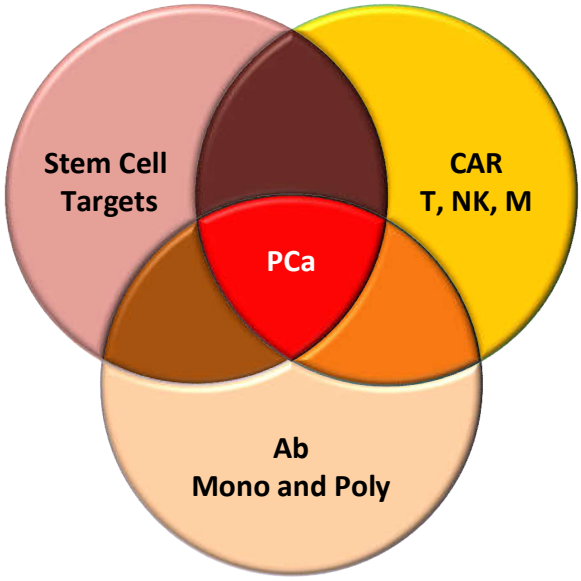
If one were able to target multiple cancer cell targets then perhaps one can obtain better results with little accompanying morbidity. We consider such a model below. Name we could suspect that as the number of cancer cell markers that are used increases the specificity increases and the chance of causing related morbidity decreases. Here we plot effectiveness and morbidity versus the number of markers. This is a purely speculative representation but it is the basis of the details we will address herein. We believe the tools that are available for accomplishing this result are readily available yet they require substantial clinical validation as well as demanding the possibility of patient specific targeting.



1.4 APPROACH

It may be useful to understand our approach in this report. This is neither a review nor a primary research effort. It is rather an attempt to holistically address a question; what are the future options in dealing with prostate cancer and why? Thus we present basics from the literature in a systematic manner hopefully to evoke an understanding of what could be next. As we noted previously this is akin to puzzle design. Our intent to present that image of a possible future set of prostate cancer therapeutics. We then assemble a set of pieces to this puzzle and assemble them in a hopefully coherent manner. There is fact and speculation herein. But fundamentally we hope that we have assembled the puzzle pieces in an adequate manner to lay out both the image and the path.

Namely we attempt to select a set of pieces and then tie them together into a single vision forward as shown below:



We do so by following the literature in each area and then selecting the key contribution reflective in each as pieces to the puzzle and then assemble a more cohesive and holistic vision of what can be accomplished. We thus examine this as a system of elements and connect them together as a whole.

1.5 OVERVIEW

This report thus addresses the above issues. Specifically:

1. PCa stem cell construct is discussed and placed within the overall construct of the development and control of PCa.

2. A detailed analysis of the current understanding of cancer cell surface markers is presented. This is to establish putative targets for immune therapies.

3. Antibody, Ab, approaches to controlling cancers is discussed at length and it includes an expansive discussion of the more recent complex Abs and their efficacy.

4. We then discuss NK cells and other immune cells as vehicles to target the surface markers unique to PCa.

5. We expand this to CAR constructs and examine the use of a variety of such CARs including the complex 4th generation CARs.

6. Finally we make several observations on what has been presented as well as extensions to the material discussed.

2 PCA STEM CELLS

There has been a great deal of work on stem cells. We may think of such cells as being part of the embryo, and in the placenta at birth. They are thought of as the universal cell generator. Theoretically the stem cell should become whatever cell type we may want it to be. In a narrower sense there may be a variety of localized stem cells, namely cells which replenish local cells which are worn away such as on the skin or in the colon. It is not the mature cells which do the reproducing but it is the few stem cells which reside in say the basal layer of the skin which reproduce and create off spring which are just plain old keratinocytes.

We examine in some detail the prostate stem cell, and in turn we generate the ability to consider the cancer stem cell issue in broader detail.

The focus is on stem cells. It does not address the pathways which are different or activated. That in itself is a critical question. Namely what differentiates a stem cell from a mature non stem like cell when we examine the pathways? Thus when looking at PCa we see that pathway changes are then most likely pathway changes in the stem cell alone, yet if the agglomeration of stem cells is such that the non-stem constituents reflect the genetic makeup of the stem cell, then we would expect some parity in pathway dynamics. This will be an issue we examine in a later report.

The cancer stem cell theory has been developed over the past decade or so. For many years the theory was that cancer was clonal, namely one single cell was at fault and its progeny were the direct result of that genetically modified parent, a single parent, and that as the cancer evolved there may be increased genetic defects but again all were from a single parent.

Cancer stems cells are a construct which predicates the development of mature cells in a cell line as coming from a set of stem cells, akin to the blood cells arising from the bone. In contrast to the linear model of Vogelstein, say in the colon, the epithelial cell of the colon wall has some genetic disruption, and after multiple disruptions this epithelial cell becomes cancerous, dividing without bounds and failing to remain where is was supposed to. Typically an adenoma develops which after the final genetic hit becomes an adenocarcinoma.

For example, we have examined the prostate cancer cell, and in so doing have used a non CSC model, namely it is a basal or luminal cell which becomes genetically changed. If however we are wrong and there is an equivalent prostate cancer stem cell, as some have conjectured, then management of cancer of the prostate is quite a different thing. As we have expressed before, if one has diffuse HGPIN in the prostate and then after several high density prostate biopsies it disappears, is that inferentially valid for a prostate CSC?

The cancer stem cell construct is fundamentally different. It is not a mature cell which takes the genetic hits but the stem cell. The malignant stem cell acts almost as a force at a distance, and can impact other cells as the stem cell itself can reproduce, albeit at a somewhat slower rate than what it may influence.

Arguably if one can remove the stem cell then one removes any future malignancy, even to the extent of having other cells enter apoptosis for failure of having an active stem cell.

As Weinberg notes, there is the theory of clonal development of cancer which states that the cancer cells are pluripotent and have developed from a single source and that they have the capability of reproducing and do so in an autonomous manner[3]. Then there is the theory of the cancer stem cell, the theory which states that there is the equivalent of a stem cell as we know in blood cells, which have the capability but that the majority of malignant cells do not necessarily have that capacity.

The NCI presents an excellent summary of Cancer stem cell, CSC, research[4]:

The theory of the cancer stem cell (CSC) has generated as much excitement and optimism as perhaps any area of cancer research over the last decade. Biologically, the theory goes, these cells are distinct from the other cells that form the bulk of a tumor in that they can self-perpetuate and produce progenitor cells, the way that traditional stem cells do. The progenitors' job is then to repopulate tumor cells eradicated by treatments such as chemotherapy or radiation.

But for all the attention and fanfare CSC research has received, the findings reported to date are far from clear-cut, investigators acknowledge. For example, most of the studies that have identified human CSCs have used mouse xenograft assays and cells from only a small number of human tumor samples, making it difficult to draw firm conclusions. In addition, other researchers haven't always been able to replicate initially reported findings. And while these tumor-initiating cells, as they are also called, have been described as being a rare class, several studies have found that the number of cells that can form tumors in these mouse experiments is actually quite large, suggesting that perhaps CSCs aren't such a privileged breed.

As we shall discuss herein, the CSC does not yet have a steady state definition or description. Furthermore it is also difficult to tag and identify. In the above definition, there is the issue of what makes the stem cell different and how many are there and how do we identify it. The CSC is in one sense the single cell which can regenerate a full cancer growth. But does that mean in vivo or in vitro or both? Murine models have been used extensively but there are serious questions regarding their extensibility.

We shall discuss some of these issues in this report. Now the NCI goes on to say:

In other words, the idea of just what cancer stem cells are, and their role in different cancers, appears to be changing.

"The [stem cell] model has not been adequately tested in most cancers," said Dr. Sean Morrison, who directs the Center for Stem Cell Biology at the University of Michigan. "I think

[3] Weinberg, Cancer, pp 416-417.

[4] http://www.cancer.gov/ncicancerbulletin/072710/page4

that there are some cancers that do clearly follow a cancer stem cell model…But it will be more complicated than what's been presented so far."

They continue by noting a significant conclusion of the CSC theory, the fact that the CSC is the controlling cell, not just any cell. Specifically they state:

Unlike the random or "stochastic" model dominant in cancer research, which holds that nearly any cancer cell has the potential to form a tumor, the cancer stem cell model is one of a hierarchical organization, with the pluripotent cancer stem cell sitting ready and able to amass all of the components of the original tumor.

It's also thought, with some experimental evidence to support it, that CSC pluripotency allows these cells to adapt and to resist chemotherapy, radiation therapy, and even current molecularly targeted therapies. If true, then these treatments may not harm the most lethal tumor cells, those that can lead to a recurrence with the production of a new set of progenitors.

Despite numerous studies published in the last 16 years that identified CSCs for different cancers—including colon, brain, pancreatic, and breast cancer—the consensus among researchers seems to be that the evidence is strongest for the first cancer in which a population of tumor-initiating cells was discovered, acute myeloid leukemia (AML), as well as for other blood cancers.

The above has substantial positive and negative impact. A single stem cell may control everything, for a while. If however it undergoes mitosis then we may have many stem cells. Or we may keep a single one. For example if a stem cell in mitosis reproduces a single stem cell plus a non-stem cancer cell, then we maintain single CSCs, while we multiply the malignant non CSC cells. However, if the CSC in mitosis just multiples itself for a while, then we end up with a collection of very powerful and spreadable bombs of CSCs.

The NCI also continues:

"The reason why it's so much stronger for hematologic malignancies are because hematopoiesis research goes back 40 or 50 years and it's very stem cell-based," said Dr. Jean Wang, a stem cell researcher at the University of Toronto. "Whereas in solid tumors, there's less of a foundation for identifying the normal cellular hierarchies and for [cell-surface] markers that identify different populations of cells like stem cells and progenitors."

The above comment has some merit but one must also recognize that the hematopoietic cells are fundamentally generated in a specific location, the bone, and there may very well be no such locations specificity for the many other cells we are considering. Nevertheless, we continue:

Even so, Dr. Wang believes the existence of CSCs is pretty well demonstrated for breast and brain cancers. But, she cautioned, "I don't know if it applies to all cancers. In a lot [of cancers] it does seem to apply. But most of the markers we have right now are still very rough."

Despite the evidence for CSC-like cells in a growing number of cancers, the theory clearly has its skeptics, who point to problems such as shortcomings in the mouse xenograft assay and the variable specificity of the cell-surface markers used to demarcate a CSC from a non-CSC.

"I still feel that it's a concept yet to be proven," said Dr. Barbara Vonderhaar, who, along with colleagues in NCI's Center for Cancer Research, recently published a study identifying a population of CSC-like cells in estrogen receptor-negative breast cancer. "It's certainly a good idea, but it's only a hypothesis at this point. We still don't have definitive proof that cancer stem cells exist."

The CSC concept is "a work in transition," said Dr. William Matsui, from the Johns Hopkins School of Medicine, whose lab studies the role of stem cells in hematologic cancers. "To me, as a clinical person, the ideal model is one where you can find something that is going to work in humans. We're far from that."

The existence of CSCs in PCa has been examined and as with many cancers is still open for discussion. However as we shall discuss later the CSC model does have certain interesting uses in the progression and metastasis of cancer.

For example:

Cell Proliferation: If we assume that the CSC is the dominant cell that proliferates and all others do not, albeit being cancer cells themselves, then the growth of PCa in terms of cells is complex but one can then more easily explain indolent PCa.

Metastasis: We know that metastasis occurred by lymphatic and hematological means. However PCa cells, non-CSC PCa cells may break loose and yet not result in classic metastasis. The issue then is one where it may be necessary for the CSC to move by these means.

Many other such issues will arise and we discuss the CSC idea here and we return to it later in the work.

Now we can view the stem cells as shown below. There is a stem cell which can give rise to a new stem cell of ultimately a Post Mitotic Differentiated Cancer Cell. The PMDC cannot replicate, whereas the stem cell can. For metastasis it is thus necessary to send out a few stem cells, not PMDC cells.

As Yun et al have noted:

Cancer stem cells (CSCs) share many characteristics with somatic stem cells, such as immortality and self-renewal. In addition to normal stem cell properties, CSCs appear to be tumor initiators and show resistance to therapies because of their quiescence.

Increasing evidence indicates that CSCs are present in the end stage of disease. Although the cell origin of castration resistant prostate cancer (CRPC) remains controversial, several studies clearly indicate the presence of CSC in CRPC.

Despite of many potential stem cell markers identified in prostate, in human prostate cancer, the CD44þ/CD24 cells have been associated with the prostate cancer stem cell. CD44 has been implicated in numerous biologic processes including cell adhesion, migration, drug resistance, and apoptosis.

Furthermore, many studies implicate CD44 in prostate cancer development and invasion in vitro and in metastatic dissemination in vivo. However, the mechanism(s) associated with elevated CD44 in prostate cancer is largely unknown. DAB2IP is characterized as a novel tumor suppressor in prostate cancer metastases by inhibiting epithelial-to-mesenchymal transition .

Besides, our recent study showed that DAB2IP had a critical role in suppressing stemness through modulating CD117 transcription . In this study, we demonstrate that loss of DAB2IP expression in nontumorigenic normal prostate epithelia derived from androgen receptor-negative basal cell population also increases their tumorigenicity, stemness and chemoresistance. Unlike prostate cancer cell lines which were used in previous study , these normal prostate epithelial cell populations exhibit CD44þ/CD24 instead of CD117þ suggesting existence of another regulation mechanism.

Apparently, CD44 is not only a stem cell marker correlated with prostate cancer progression but also a driver for PCSC formation in which Wnt pathway is further identified as a key underlying mechanism in modulating CD44 expression. Based on these findings, we developed a combination strategy using Wnt inhibitor and docetaxel to target both CSC and its progeny non-CSCs respectively, to significantly enhance therapeutic efficacy of CRPC. Overall, this study provides strong evidence of CSC in CRPC and offers a new therapeutic regimen for CRPC.

Thus we first examine the stem cell construct then we examine the specified surface markers.

2.1 THE STEM CELL PARADIGM

The first issue is a definition of a stem cell[5]. We may understand stem cell from the hematopoietic stem cells found in the bone which give rise to a variety of blood cells and other types of cells. In fact almost all cells in the body which require some form of replenishment have such stem cells. Consider the skin. The basal layer has stem cells to generate the keratinocytes. In fact it may be argued that melanocytes have their own stem cells as well.

Now Rajasekhar et al note:

The stem-like TICs do not express androgen receptor and prostate-specific antigen. The spheres express distinct characteristics and are multipotent as the sphere-cell-derived tumours (sphere tumours) reconstituted histopathological features and immunophenotypes of the original OT-human prostate CWR22 tumour (parent tumour) and closely mimicked the features of freshly obtained patient prostate tumour.

[5] See Shackleton et al

Thus, these stem-like sphere cells do not express key markers of prostate cancer, including AR, prostate specific antigen (PSA), NKX3.1 (androgen/AR-regulated transcriptional coactivator) and cytokeratin-18 (CK18, differentiated luminal cell enriched marker).

In contrast, cancer stem cell and cell-proliferation-associated markers MET receptor kinase, Musashi-1, inhibitor of differentiation 1, phospho-histone 3 and Ki67 are selectively enriched in these spheres relative to tumours.

We also found that the AR-/PSA-negative stem-like sphere cells are of basal epithelial-like cell type based on the expression of E-cadherin, cytokeratin-5 (CK5) and SOX9 and lack of expression of markers of myoepithelial cells (smooth muscle actin), mesenchymal cells (vimentin) and neuroendocrine cells (synaptophysin). However, these sphere cells also lack detectable expression of basal cell enriched, p63 and its polarity associated, zonula occludens-1 (ZO-1).

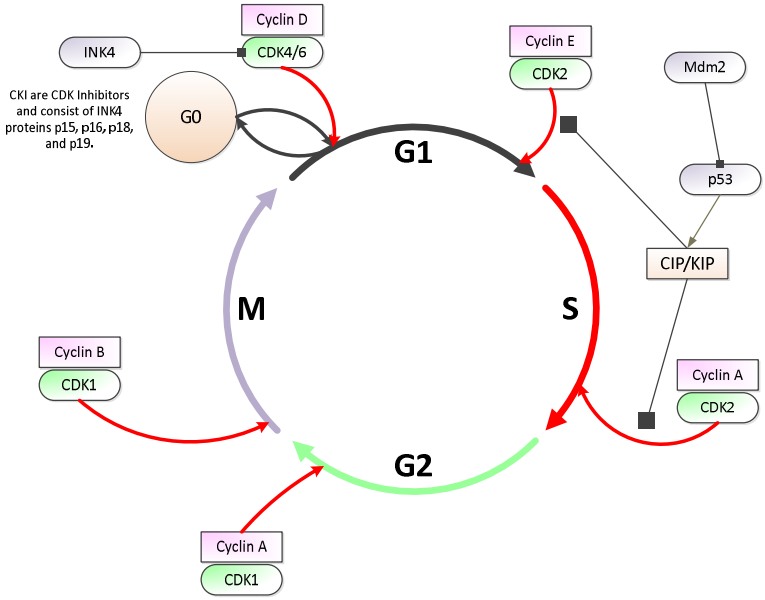
Thus, these sphere-forming TICs represent a distinct cancer stem-like cell compartment in the human prostate tumour. Human prostate TICs express novel pluripotent cell markers. By fluorescence-activated cell sorting (FACS), we investigated novel cell surface marker expression that could facilitate prospective isolation of the TICs in the human prostate CWR22 OT-tumours. First, we confirmed the expression of a set of markers known to associate with stem-like tumour cells in other epithelial cancers, including human prostate tumours17,27. They included epithelial cellular adhesion molecule (EpCAM), hyaluronic acid receptor (CD44) and integrins.

Prostate tumour cells expressing these markers displayed increased sphere-formation capacity as compared with unsorted total tumour cells or cells expressing β4-integrin.

Except for CD44, the percentage of cells expressing these markers (EpCAM, α2-integrin for example) was consistently enriched over sequential passages of sphere cells. The above markers are associated with many different cell types including stromal and/or interstitial cells in the prostate and in other organs.

Therefore, to facilitate precise identification of stem-like TICs, we investigated novel markers such as the tumour rejection antigen, TRA- 1-60, a cell surface epitope of human embryonic, embryonal germline and teratocarcinoma stem cells28. Sphere-forming cells and a subset of tumour cells express TRA-1-60, related pluripotent stem cell markers such as TRA-1-81 and stage-specific embryonic antigens such as SSEA1, SSEA4 for example. TRA-1-60 or TRA-1-81-positive tumour cells formed spheres more efficiently than cells positive for other known markers, and the TRA-1-60-positive tumour cells were moderately more efficient in tumour induction than the primary sphere cells

Cells are reproducing via the cell cycle as we show below and discuss in Appendix B. With a stem cell, it is only that cell which does the mitotic division; all other cells are just mature functioning cells subject to normal cell death or apoptosis.



The question is however, which cells. Which cells are the stem cells? Are all cells reproducing or just some select class of cells. The concept of stem cells makes the issue one of a small select group of cells. These are the stem cells.

As Alberts et al state (pp 1417-1421):

Humans renew the outer layers of their epidermis a thousand times over in the course of a lifetime. In the basal layer, there have to be cells that can remain undifferentiated and carry on dividing for this whole period, continually throwing off descendants that commit to differentiation, leave the basal layer, and are eventually discarded.

The process can be maintained only if the basal cell population is self-renewing. It must therefore contain some cells that generate a mixture of progeny, including daughters that remain undifferentiated like their parent, as well as daughters that differentiate. Cells with this property are called ***stem cells****.*

They have so important a role in such a variety of tissues that it is useful to have a formal definition. The defining properties of a stem cell are as follows:

1. It is not itself terminally differentiated (that is, it is not at the end of a pathway of differentiation).

2. It can divide without limit (or at least for the lifetime of the animal).

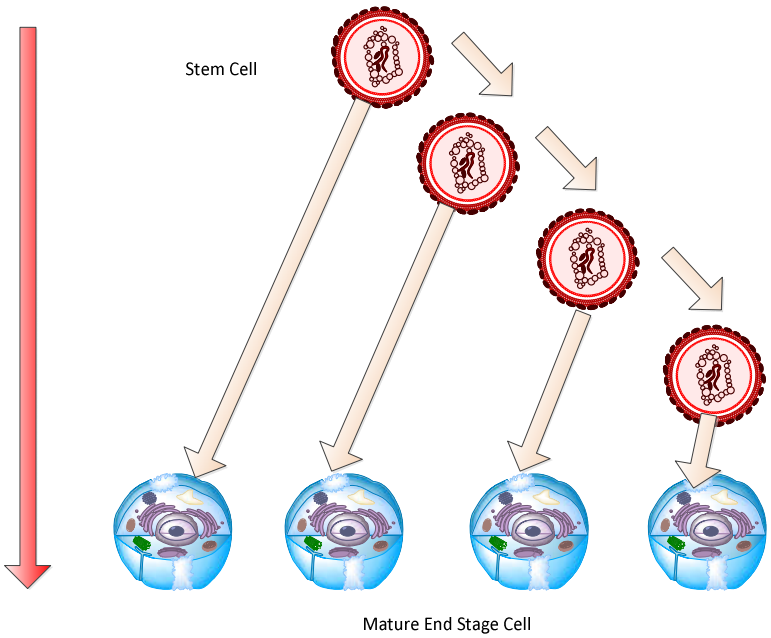
3. When it divides, each daughter has a choice: it can either remain a stem cell, or it can embark on a course that commits it to terminal differentiation.

Stem cells are required wherever there is a recurring need to replace differentiated cells that cannot themselves divide. The stem cell itself has to be able to divide—that is part of the

definition—but it should be noted that it does not necessarily have to divide rapidly; in fact, stem cells usually divide at a relatively slow rate.

2.2 EXAMPLES

We present below a simplified example of a specialized stem cell. The stem cell is the only one of its kind to divide. The mature cells do not generally divide; they are just functional and proceed to mature. The stem cell always produces at least one of its own kinds, another stem cell, and then one of the mature like cells. Note the initial stem cell. In this example we allow it to divide and produce one stem cell and one maturing cell. Thus at some point this process just keeps the number of stem cells constant but can produce an ever growing number of maturing cells.



Now when we examine the above we can see that if the stem cell divides once every hour, and the life of a mature cell is say 24 hours, then we have a growth effect. We must have a cell stability of one replenishment per one destroyed. During a growth state however, the stem cells are reproducing quickly and cells are added. The stem cell responds to surface stimulants to enter into cell cycle production.

As Tang et al state:

Normal adult stem cells (SC) have several fundamental properties: they are generally very rare, can self-renew, have tremendous proliferative potential but normally (i.e., in their niches) are quiescent, and can differentiate along one or several different cell lineages.

The most defining property of a SC is its ability to self-renew while being able to differentiate into all different lineages of progeny and even to reconstitute an organ, as exemplified by a single hematopoietic SC (HSC) to reconstitute the whole blood and rescue an irradiated mouse.

SC development is a continuous and dynamic process, in which cells with distinct self-renewal, proliferative, and differentiation abilities may co-exist.

For example, mouse HSC are heterogeneous populations of cells containing long-term HSC (LT-HSC), which can sustain life-long self-renewal and reconstitution, and short-term HSC (ST-HSC), which can sustain self-renewal and reconstitution for only 8 wk. The ST-HSC generate multi-potent progenitor (MPP) cells exhibiting only limited self-renewal capacity, which then further develop into lineage-restricted progenitor (or precursor) cells that have lost self-renewal ability.

Although this paradigm of LT-HSCST-HSC early progenitors (MPP) late progenitors differentiated cells in mouse bone marrow can, in principle, be applied to other SC developmental processes, in reality, little is known about most tissue SC lineages and we often name the subsets of cells in a specific tissue/organ with certain self-renewal and differentiation abilities simply stem/progenitor cells. Such is the case with the putative prostate epithelial stem and progenitor cells.

Consequently, throughout this review, we shall frequently use the term '(prostate) stem/ progenitor cells.'

The above feature of maturing into various lineages is clearly seen in blood cells but one may question just where it functions say in prostate cells. Is there a single stem cell which generates either a basal or luminal cell or if so where does it reside, and how does this differentiation occur? This is the point made by Tang et al towards the end of the above quote.

Cancer stem cells are a variant of the benign stem cell. Namely a cancer stem cell is a cell which behaves like a stem cell in terms of cell proliferation but now has genetic changes which reflect malignant behavior. In an NIH report the authors define cancer stem cells as follows:

A consensus panel convened by the American Association of Cancer Research has defined a CSC as "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor." It should be noted that this definition does not indicate the source of these cells—these tumor-forming cells could hypothetically originate from stem, progenitor, or differentiated cells.

As such, the terms "tumor-initiating cell" or "cancer-initiating cell" are sometimes used instead of "cancer stem cell" to avoid confusion. Tumors originate from the transformation of normal cells through the accumulation of genetic modifications, but it has not been established unequivocally that stem cells are the origin of all CSCs.

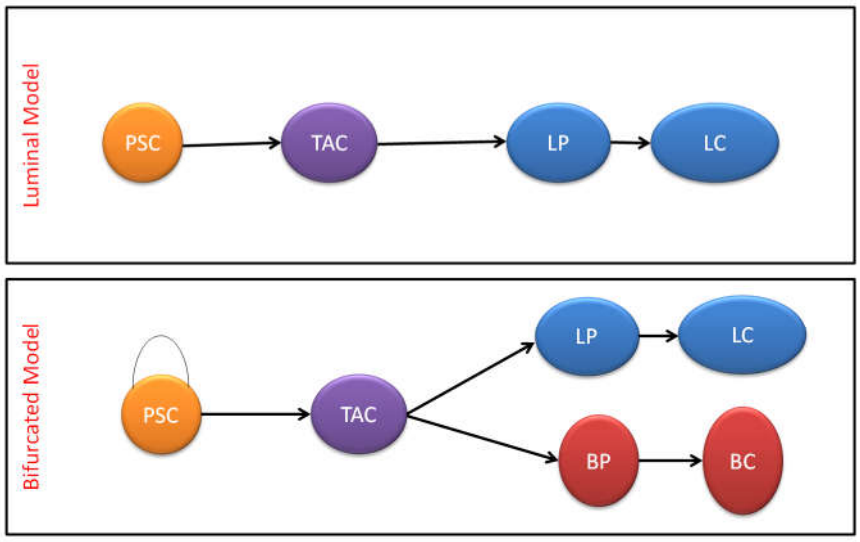
The CSC hypothesis therefore does not imply that cancer is always caused by stem cells or that the potential application of stem cells to treat conditions such as heart disease or diabetes, as discussed in other chapters of this report, will result in tumor formation. Rather, tumor-initiating cells possess stem-like characteristics to a degree sufficient to warrant the comparison with stem cells; the observed experimental and clinical behaviors of metastatic cancer cells are highly reminiscent of the classical properties of stem cells.

The stem cell theory, and there seems now to be significant evidence of its validity in prostate cancer, is principally that the clonal theory has merit to a point but that the development is more complex and the cancer stem cell plays a critical role in fostering growth of the cancer cells, most of which has less aggressive a growth characteristic if any at all.

2.3 PROSTATE SPECIFIC

Lawson and Witte present a recent overview of this concept as applied to the prostate and PCa. Recent studies apparently indicate that the cancer stem cells, CSC, are necessary to sustain later stages of the development of the malignancy. Only a small subpopulation of the cancer cells, the CSC population, has a demonstrated ability to maintain the malignancy as well. Lawson and Witte present two theories of this CSC process. One is called the stochastic theory which is that all cells are equally malignant. The other theory, the one for CSC, called the hierarchical theory is that only the CSC has the ability to multiply.

The CSC or in this case the PSC, prostate stem cell, yields a TAC, or transition amplifying cells, then yield progenitor cells, LP or BP, and then finally a luminal or basal cell. This is slight contrast to the Goldstein model. This model applies for both benign as well as cancer cells, at least as viewed by Lawson and Witte.



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Now if one looks at the CSC theory, then we see a CSC has progeny, and yet those progeny may not have the ability to multiply. Thus the explosive exponential growth of cancer is not as clear in a CSC model, because almost all of the progeny of the CSC are no reproducing progeny. Thus the growth models for a CSC based malignancy are more complex and are dependent on limited CSC reproduction and non-CSC reproduction.

However the CSC model also argues for there being some CSC support for the progeny which are not CSC. The dynamics of cell growth then becomes quite complex here, for the stem cells replicate themselves at a slow rate but are replicating other cells at a higher rate. However the other cells do not replicate themselves they just go through a standard cell process. If the cells are benign then they go through apoptosis as seen in red blood cells and the skin keratinocytes.

We quote Lawson and Witte as follows:

Models of prostate epithelial differentiation. The traditional model for prostate epithelial differentiation proposes that PSCs residing in the basal cell layer give rise to intermediate, transit-amplifying cells that produce large numbers of terminally differentiated secretory luminal cells …. This model implies a linear differentiation scheme in which basal and luminal cells comprise one lineage and basal cells are essentially luminal cell progenitors …

This hypothesis is supported by the existence of cells of intermediate phenotype that express both basal- and luminal cell–specific cytokeratins in both fetal and adult stages of prostate development … Intermediate cells can also be identified in in vitro cultures of primary prostate epithelium … Several studies have also suggested basal cells can differentiate into luminal cells in vitro … Alternative theories for prostate epithelial differentiation propose basal and luminal cells may represent separate epithelial lineages … This is similar to prevailing models for epithelial differentiation in the mammary gland, a tissue that is anatomically and functionally analogous to the prostate …

Now there have been several others who have examined the stem cell model for PCa. Another of recent merit is that of Hurt et al. They summarize their work as follows:

Recent evidence supports the hypothesis that cancer stem cells are responsible for tumor initiation and formation. Using flow cytometry, we isolated a population of CD44+CD24- prostate cells that display stem cell characteristics as well as gene expression patterns that predict overall survival in prostate cancer patients. CD44+CD24- cells form colonies in soft agar and form tumours in NOD/SCID mice when as few as 100 cells are injected.

Furthermore, CD44+CD24- cells express genes known to be important in stem cell maintenance, such as BMI-1 and Oct-3/4. Moreover, we can maintain CD44+CD24- prostate stem-like cells as non-adherent spheres in serum-replacement media without substantially shifting gene expression. Addition of serum results in adherence to plastic and shifts gene expression patterns to resemble the differentiated parental cells.

Thus, we propose that CD44+CD24- prostate cells are stem-like cells responsible for tumor initiation and we provide a genomic definition of these cells and the differentiated cells they give rise to. Furthermore, gene expression patterns of CD44+CD24- cells have a genomic signature that is predictive of poor patient prognosis. Therefore, CD44+CD24- LNCaP prostate cells offer an attractive model system to both explore the biology important to the maintenance and differentiation of prostate cancer stem cells as well as to develop the therapeutics, as the gene expression pattern in these cells is consistent with poor survival in prostate cancer patients.

Jordan et al characterize cancer stem cells as having three characteristics:

1. Self-Renewal: at the end of mitosis of the stem cell, either one or both retain all the characteristics of the parent. The stem cell goes through a mitotic doubling and when it does it always retains one or two stem cell daughters.

2. Capability to generate multiple lineages. This means that a stem cell can generate offspring which can become anyone of many cell types.

3. Potential to proliferate extensively. The cell can keep replicating, it has no limitation within reason and thus contains the elements ultimately for metastasis.

A normal stem cell may mutate to a cancer stem cell or a normal progenitor cell may morph back to a cancer stem cell.

As Delarbra et al state:

Although monoclonal in origin, most tumors appear to contain a heterogeneous population of cancer cells. *This observation is traditionally explained by postulating variations in tumor microenvironment and coexistence of multiple genetic subclones, created by progressive and divergent accumulation of independent somatic mutations.*

An additional explanation, however, envisages human tumors not as mere monoclonal expansions of transformed cells, but rather as complex tridimensional tissues where cancer cells become functionally heterogeneous as a result of differentiation.

According to this second scenario, tumors act as caricatures of their corresponding normal tissues and are sustained in their growth by a pathological counterpart of normal adult stem cells, cancer stem cells.

The statement starts with the accepted monoclonal hypothesis and then departs to a polyclonal alternative view. It retains the CSC, cancer stem cell, paradigm for solid tumors as well. In the context of HGPIN we see a change in the cells and we have heard the argument that they have made one or several of the unchangeable steps towards PCa. Thus using the CSC theory one would expect that it would be from one or several of these cells that PCa would arise. In addition, we could assume that there are no unique pathway mutations or changes which result in PCa but a plethora of them. Simply stated, cancer is complex, it finds ways to migrate forward no matter what the path.

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or changes which result in PCa but a plethora of them. Simply stated, cancer is complex, it finds ways to migrate forward no matter what the path.

A recent study by Deleyrolle et al has focused on the stem cell and its dynamics[6]. The reviewers state:

The method, published in the online journal PLoS ONE in January, may rev up efforts to develop stem cell therapies for Alzheimer's, Parkinson's and other diseases. It may also help get to the root of the cancer-stem cell theory, which puts forth the idea that a tiny percentage of loner cancer cells gives rise to tumors.

"Math is going to be the new microscope of the 21st century because it is going to allow us to see things in biology that we cannot see any other way," said Brent Reynolds, Ph.D., an associate professor of neurosurgery at UF's McKnight Brain Institute and a member of the UF Shands Cancer Center. "Stem cells and the cells that drive cancer may be as infrequent as one in 10,000 or one in 100,000 cells. The problem is how do you understand the biology of something whose frequency is so low?"

Inspired by a 2004 essay by Joel E. Cohen, Ph.D., of The Rockefeller University and Columbia University that described the explosive synergy between mathematics and biology, Reynolds and postdoctoral associate Loic P. Deleyrolle set out to build an algorithm that could determine the rate stem cells and cancer stem cells divide.

High hopes to treat or prevent diseases have been pinned on these indistinguishable cells, which are often adrift in populations of millions of other cells. Scientists know stem cells exist mainly because their handiwork is everywhere — tissues heal and regenerate because of stem cells, and somehow cancer may reappear years after it was thought to be completely eliminated.

Nature has an interesting poster on the cancer stem cell, CSC[7]. The poster states:

The concept of the cancer stem cell (CSC) has taken off rapidly over the past 10 years. CSCs are cells with properties that are similar to those described for tissue stem cells: self-renewal and asymmetric division resulting in the generation of daughter cells destined to differentiate, enabling the regeneration of a tissue. Initial research into the properties of CSCs was based on identifying and verifying markers of this subset of cancer cells.

However, most studies have now moved on to understanding the biology of CSCs and the cancers in which they maintain tumour growth, as well as how and why they are able to serially generate a tumour. It is thought that a key element regulating the biology of stem cells is their niche — cells and extracellular matrix that support self-renewal and survival. As we begin to understand the pathways that are crucial for the properties of CSCs, including signals provided by the niche, we will hopefully be able to effectively target this cell population.

[6] http://www.eurekalert.org/pub_releases/2011-01/uof-gfm012011.php

[7] http://www.nature.com/nrc/posters/cancerstemcells/csc_poster.pdf

Linked to the identification of CSCs is the cell of origin. These are cells that when mutated are able to give rise to a tumour. Although these cells may share properties with CSCs, in most cases it is not yet clear whether these cells are one and the same. This poster highlights some of the recent findings regarding the biology of CSCs and the identification of cell types from which cancers can arise.

As regards to prostate cancer they state:

In the normal prostate, epithelial cells with tissue-regenerating capacity that are Sca1+, CD49fhi, TROP2hi, CD44+, CD133+ and CD117+ (mouse) or CD133+, CD44+, CD49fhi and TROP2+ (human) seem to reside in the basal layer of the prostate.

However, studies in mice indicate the existence of luminal cells with progenitor characteristics that can regenerate the prostate after androgen withdrawal. As castration resistance is also a property of basal stem cells in the prostate, it suggests a complex cellular hierarchy.

Studies in mice indicate that prostate tumours can arise after transformation of basal stem cells and luminal progenitor cells. A subset of cells that are CD133+, a2b1 + and CD44+ and have basal cell characteristics have been shown to be tumorigenic, but whether these cells can serially propagate tumours in mice has yet to be verified.

Again and interesting experiment can be performed:

1. Take biopsies from N men with HGPIN diagnosed on initial biopsies. Perform sampling from say 20 cores.

2. Wait 9 months, and rebiopsy, again with near saturation cores, 20+... There are three possible outcomes:

a. HGPIN remains
b. PCa has been determined
c. HGPIN regresses and only benign cells are left

3. The question is why did (c) above happen? What percent of the HGPIN have regressed? If the percent of HGPIN that have regressed equals the probability of having actually excised the cancer stem cell or cells, we can calculate this, then by chance we have removed the CSC from the HGPIN and this would affirm its existence by inference.

Now a similar article appears in Science which speaks to colon cancer and the cancer stem cell theory[8]:

In normal colon tissue, intestinal stem cells (ISCs) that reside at the base of mucosal wells, named crypts, expand through mitosis and move upward toward the crypt tip. The cells then undergo cell cycle arrest and terminal differentiation, finally becoming the mucosal epithelium

[8] http://stm.sciencemag.org/content/3/81/81ec64.short?rss=1

of the colon. In the recent study, the investigators identified in mouse ISCs a gene signature that was specifically marked by high expression of the ephrin type-B receptor 2 genes (Ephb2), which encodes a receptor tyrosine kinase, the leucine-rich repeat–containing G protein–coupled receptor 5 gene (Lgr5), which encodes a G-coupled protein receptor of unknown function, and ~50 other genes.

This gene signature also defined a specific population of stem-like cells at the base of colorectal tumor structures in mice that were morphologically similar to normal mouse intestinal crypts. The authors then similarly inspected tumor samples from 340 colorectal patients and discovered a 10-fold increase in the relative risk of recurrence in patients whose tumors displayed high expression of the human counterparts of the mouse ISC genes, relative to patients whose tumors showed low expression of these genes.

To test whether the mouse colorectal tumor cells with the ISC gene signature were cancer stem cells; the investigators isolated the cells and introduced them into an immunodeficient mouse model. The stem-like cancer cells demonstrated both a tumor-initiating capacity and self-renewal capability in vivo.

These findings pinpoint potential markers that may allow a clinician to predict a patient's future with respect to recurrence. These differentially expressed genes also may give rise to therapeutic targets that quell cancer stem cells.

What is clear is that the CSC is becoming a viable model for understanding cancer at another level.

We first relook at the progression and regression dynamics. The key driver for the analysis herein has been the regression often seen in HGPIN. Knowing that most likely the methylation of GSTP1 has given rise to development of PIN we then ask what gives rise to its regression and why have the HGPIN cells themselves not only stopped growing but have disappeared. Again we have seen this in melanomas, and this is also the Rosenberg effect in certain sporadic cancer regressions.

To look more closely we first return to the stem cell model for cancer which we developed earlier. The stem cell theory states that there are a certain number of cancer stem cells which in turn may replicate themselves but also create what are termed post mitotic differentiated cells. Not really stem cells but cells which exhibit the phenotypic characteristics of a cancer cell. One of the questions one may pose is do these PMDC exhibit a different genotypic character as well or are they controlled by some epigenetic factors.

Now we can also see as Weinberg has noted (Weinberg p 419) that a progression may occur in a somewhat more complex mechanism as we depict below. Now from the stem cell arise Transit Amplifying Cells and then the PMDC.

Now in reality there may be multiple genetic hits which give rise to the stem cell, the pluripotent self-replicating core of a cancer. The Figure below provides a generic profile, namely we may

see many genetic changes, some leading to cancer as in mutation 3 below and others just wandering off into self-replicating cells but not with a malignant tendency.

Finally when we return to the HGPIN model we see the benign cell migrating to a dysplasia, say HGPIN, and then to a malignant cell, but then there is the regression back to a benign cell. The question is then; what pathway elements take us one way and what elements take us back. And what happened to the dysplastic cells? Did they just die, apoptosis, or were they scavenged?

Wang and Shen have written a quite useful review of the cancer stem cell thesis for prostate cancer. There is no definitive conclusion but the review covers a wide path through what has been accomplished to date.

Now Moltzahn and Thalmann have noted regarding PCa stem cells:

The prostate consists of 3 cell types. Basal cells are relatively undifferentiated, androgen-independent, cells. They express CK 5 and 14, CD44 and p63, but no or only low androgen receptors (AR-), PSA and PAP. Secretory luminal, and glandular epithelial cells are differentiated cells of the mature prostate with androgen-receptor (AR+), PSA, PAP, CK 8 and 18 expression. The neuroendocrine cells appear to be androgen-independent and fully differentiated cells containing chromogranins without expression of AR or PSA . The existence of physiological stem cells in prostate was deduced from the finding that androgen ablation leads to involution of the androgen-dependent compartments of the prostate, but that subsequent androgen replacement results in total reconstitution of the organ. Based on these observations, Isaacs and Coffey developed a PSC model .

According to this model, androgen-independent stem cells give rise to progenitor cells that are androgen-sensitive, but not androgen-dependent, which then, under the influence of androgen, differentiate into androgen-de- pendent cells of the prostate epithelium.

Following Isaacs and Coffey's theory the PSC were thought to reside in the basal cell layer, as it remains intact during androgen ablation-caused prostate involution. Prostate CSC CSCs in P-Ca are not well understood yet. There exists conflicting data for putative markers, the cell of origin as well as the location of P-Ca stem cells (PCSC) within the organ. However, ongoing research in mice and human provides convincing evidence that P-Ca follows the hierarchical model .

Markers used to study PSC and PCSC Most studies investigating PCSC used established cell lines, primary tumors or xenografts in immuno-deficient mic.

Multiple markers for the characterization of PSCs and PCSCs have been proposed, including cell surface markers, marker of self-renewal, pluripotency and markers of resistance to therapy .

Collins isolated rare cells from ***human primary P-Ca using the combination of the surface markers CD44+α2β1integrinhighCD133+ that were able to self-renew in vitro****. Using the same combination prostate CSCs were isolated from the cell line DU145. Interestingly CD44, a*

glycoprotein involved in cell-cell interactions, cell adhesion and migration, has been identified as a marker of stemness of CSC for many different organs/cancer .

Patrawala revealed that CD44+ P-Ca cells from xenograft human tumors were enriched in tumorigenic and metastatic progenitor cells compared to CD44- cells . Hurt demonstrated the tumor forming ability of CD44+CD24− prostate stem-like cells isolated from LNCaP cell line was after the injection of as few as 100 cells in NOD/SCID mice .

Holoclones from the PC3 P-Ca cell line were shown to contain cells expressing high levels of CD44, α2β1 and β-catenin, and could initiate serially transplantable tumors after subcutaneous injection . CD133 has been identified as CSC marker for a variety of malignant tumors . In prostate, CD133+ cells were demonstrated to be able to possess a high in vitro proliferative potential and to reconstitute prostaticlike acini in immunocompromised male nude mice . However, recent studies suggest that CD133- cells in certain human tumors also possess tumorigenic activity after serial transplantation in NOD/SCID mice .

Aldehyde dehydrogenase (ALDH1A1) acts in retinoic acid signaling, has important function in SC self-protection and high ALDH activity was correlated with the stem/progenitor cell state . For P-Ca it was found to be positively correlated with Gleason score and pathologic stage, and inversely associated with patient survival . In contrast to ALDH− cells, ALDH+ P-Ca cells showed CSC-like characteristics such as increased self-renewing and colony forming capacity and tumorigenicity. In addition, ALDH+ cells revealed an increased expression of putative P-Ca stem cell markers (CD44 and integrin α2β1) .

Yu reported conflicting data, as they found that ALDHlowCD44- cells were also able to develop tumors with longer latency periods, although with lower capacity compared to their ALDHhighCD44+ counterparts . Investigating PSA−/loALDH+CD44+α2β1+ phenotypes Qin described these cells to be quiescent and refractory to stresses including androgen deprivation. The cells expressed stem cell genes, and were able to undergo asymmetric cell division generating PSA+ cells. Importantly they initiated robust tumor development, resisted androgen ablation and harbored highly tumorigenic castration-resistant PCa cells. In contrast, the PSA+ PCa cells possessed more limited tumor- propagating capacity, underwent symmetric division and were sensitive to castration . Lin-,Sca1+; CD49fhi (LSChi) cells have been demonstrated to be useful for isolation of murine stem cells. In the Pten-null P-Ca model the LSChi subpopulation is sufficient for cancer initiation . Addition of CD166 further enriched sphere-forming activity of WT LSChi and Pten null LSChi. Moreover expression of CD166 is upregulated in human P-Cas, especially CRPC samples .

Nevertheless, in the Pten null mouse model downregulation of CD166 did interfere neither with sphere formation nor with progression and metastasis. Identifying the ABCG2 side population, which is associated with multidrug resistance, in combination with the surface marker CD133+/CD44+/ CD24- have been also reported to increase CSC isolation.

3 SURFACE MARKERS

We now focus on PCa surface markers. These are proteins that have some specificity for prostate and PCa cells. Their identity, perhaps in some conjunctive manner, can focus uniquely on PCa and let other cells remain unattended.

As Zijlstra and Stoorvogel have noted:

New biomarkers are needed to improve the diagnosis of prostate cancer. Similarly to healthy cells, prostate epithelial cancer cells produce extracellular vesicles (prostasomes) that can be isolated from seminal fluid, urine, and blood. Prostasomes contain ubiquitously expressed and prostate-specific membrane and cytosolic proteins, as well as RNA.

Both quantitative and qualitative changes in protein, mRNA, long noncoding RNA, and microRNA composition of extracellular vesicles isolated from prostate cancer patients have been reported. In general, however, the identified extracellular vesicle–associated singlemarker molecules or combinations of marker molecules require confirmation in large cohorts of patients to validate their specificity and sensitivity as prostate cancer markers. Complications include variable factors such as prostate manipulation and urine flux, as well as masking by ubiquitously expressed free molecules and extracellular vesicles from tissues other than the prostate.

Herein, we propose that the most promising methods include comprehensive combinational screening for (mutant) RNA in prostasomes that are immunoisolated with antibodies targeting prostate-specific epitopes…

Proteins that are exclusively expressed by PCa cells — as compared with healthy prostate epithelial cells or any other cell type — and are incorporated into prostasomes have yet to be identified. The detection of prostasomes in blood is further complicated by the concomitant presence of EVs from many other sources[9].

Nevertheless, detection of high levels of a single protein, even when ubiquitously expressed, in a total EV fraction from blood may be sufficient to detect PCa, as exemplified by a study in which the antiapoptotic protein survivin was found to be significantly increased in EVs isolated from the plasma of PCa patients compared with plasma from patients with preinflammatory benign prostate hyperplasia or healthy controls . In another study, the tumor suppressor PTEN was approximately 10-fold higher in EVs isolated from PCa patients compared with normal subjects . However, by colocalizing multiple markers on the same EV in a so called proximity ligation assay, it may be possible to detect PCa derived prostasomes in blood with greater sensitivity compared with single-marker analyses …

With this assay, PCa patients, especially those with high prostatectomy Gleason scores, were found to have elevated concentrations of prostasomes in their blood compared with healthy controls, confirming the potential of combinational analysis of blood-borne prostasomal proteins

[9] They define EVs as: *blood- and urine-borne prostate-specific extracellular vesicles (EVs, known as prostasomes) as a potential source of biomarkers for PCa*.

for PCa diagnosis. Prostasome proteins in serum may also be used to follow therapy efficacy in patients. For example, P-glycoprotein encoded by multidrug resistance protein 1 (MDR1) in blood EVs was relatively higher in docetaxel-resistant patients than in therapy-naive patients.

Zhang et al present an overview of stem cells markers for PCa. They note:

Currently there is little effective treatment available for castration resistant prostate cancer, which is responsible for the majority of prostate cancer related deaths. Emerging evidence suggested that cancer stem cells might play an important role in resistance to traditional cancer therapies, and the studies of cancer stem cells (including specific isolation and targeting on those cells) might benefit the discovery of novel treatment of prostate cancer, especially castration resistant disease. In this review, we summarized major biomarkers for prostate cancer stem cells, as well as their functional mechanisms and potential application in clinical diagnosis and treatment of patients. ...

Cancer stem cells (CSCs) were defined as cells with capacity of self-renewal and proliferation in cancer tissue . Over years, scientists have been arguing about the origin of cancer stem cells. CSCs were suggested to originate from mutated normal stem cells, from mutated progenitor cells in the process of differentiation which re-gains the characteristics of stem cells, or from mature cells that re-acquired self-renewal ability .

Various cell surface markers were used to isolate CSCs, whose proliferative potential was verified by in vitro and in vivo assays. This review summarizes recent research progress of current stem cell markers in PCa.

The authors then present the list as follows:

1. Integrins
2. CD44
3. CD133
4. CD166
5. Trop2
6. CD117
7. ALDH1
8. ABCG2
9. SOX2
10. EZH2
11. cPAcP

We shall examine these in detail here.

3.1 OVERVIEW

From Nature we have[10]:

[10] http://www.nature.com/nrc/posters/cancerstemcells

*In the normal prostate, epithelial cells with tissue-regenerating capacity that are **Sca1+, CD49fhi, TROP2hi, CD44+, CD133+ and CD117+ (mouse) or CD133+, CD44+, CD49fhi and TROP2+** (human) seem to reside in the basal layer of the prostate. However, studies in mice indicate the existence of luminal cells with progenitor characteristics that can regenerate the prostate after androgen withdrawal. As castration resistance is also a property of basal stem cells in the prostate, it suggests a complex cellular hierarchy. Studies in mice indicate that prostate tumours can arise after transformation of basal stem cells and luminal progenitor cells. **A subset of cells that are CD133+, a2b1+ and CD44+ and have basal cell characteristics have been shown to be tumorigenic, but whether these cells can serially propagate tumours in mice has yet to be verified***

We summarize a set of significant cell surface markers:

Marker	Function	Reference
CD44[11]	The protein encoded by this gene is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. It is a receptor for hyaluronic acid (HA) and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). **This protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis.** Transcripts for this gene undergo complex alternative splicing that results in many functionally distinct isoforms, however, the full length nature of some of these variants has not been determined. Alternative splicing is the basis for the structural and functional diversity of this protein, and may be related to tumor metastasis	Harris & Kerr
CD133[12]	This gene encodes a pentaspan transmembrane glycoprotein. The protein localizes to membrane protrusions and is often expressed on adult stem cells, where it is thought to function in maintaining stem cell properties by suppressing differentiation. Mutations in this gene have been shown to result in retinitis pigmentosa and Stargardt disease. **Expression of this gene is also associated with several types of cancer.** This gene is expressed from at least five alternative promoters that are expressed in a tissue-dependent manner. Multiple transcript variants encoding different isoforms have been found for this gene	Harris & Kerr

[11] https://www.ncbi.nlm.nih.gov/gene/960

[12] https://www.ncbi.nlm.nih.gov/gene/8842

Marker	Function	Reference
PSMA[13]	This gene encodes **a type II transmembrane glycoprotein belonging to the M28 peptidase family**. The protein acts as a glutamate carboxypeptidase on different alternative substrates, including the nutrient folate and the neuropeptide N-acetyl-l-aspartyl-l-glutamate and is expressed in a number of tissues such as prostate, central and peripheral nervous system and kidney. A mutation in this gene may be associated with impaired intestinal absorption of dietary folates, resulting in low blood folate levels and consequent hyperhomocysteinemia. Expression of this protein in the brain may be involved in a number of pathological conditions associated with glutamate excitotoxicity. **In the prostate the protein is up-regulated in cancerous cells and is used as an effective diagnostic and prognostic indicator of prostate cancer.** This gene likely arose from a duplication event of a nearby chromosomal region. Alternative splicing gives rise to multiple transcript variants encoding several different isoforms.	
CD117[14]	This **gene encodes a receptor tyrosine kinase**. This gene was initially identified as a homolog of the feline sarcoma viral oncogene v-kit and is often referred to as proto-oncogene c-Kit. The canonical form of this glycosylated transmembrane protein has an N-terminal extracellular region with five immunoglobulin-like domains, a transmembrane region, and an intracellular tyrosine kinase domain at the C-terminus. **Upon activation by its cytokine ligand, stem cell factor (SCF), this protein phosphorylates multiple intracellular proteins that play a role in in the proliferation, differentiation, migration and apoptosis of many cell types and thereby plays an important role in hematopoiesis, stem cell maintenance,** gametogenesis, melanogenesis, and in mast cell development, migration and function. **This protein can be a membrane-bound or soluble protein**. Mutations in this gene are associated with gastrointestinal stromal tumors, mast cell disease, acute myelogenous leukemia, and piebaldism.	Harris & Kerr

[13] https://www.ncbi.nlm.nih.gov/gene/2346

[14] https://www.ncbi.nlm.nih.gov/gene/3815

Marker	Function	Reference
E-cadherin[15]	This gene encodes a classical cadherin of the cadherin superfamily. Alternative splicing results in multiple transcript variants, at least one of which encodes a preproprotein that is proteolytically processed to generate the mature glycoprotein. This calcium-dependent cell-cell adhesion protein is comprised of five extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail. Mutations in this gene are correlated with gastric, breast, colorectal, thyroid and ovarian cancer. Loss of function of this gene is thought to contribute to cancer progression by increasing proliferation, invasion, and/or metastasis. The ectodomain of this protein mediates bacterial adhesion to mammalian cells and the cytoplasmic domain is required for internalization. This gene is present in a gene cluster with other members of the cadherin family on chromosome 16.	Harris & Kerr
CD166[16]	This gene encodes activated leukocyte cell adhesion molecule (**ALCAM**), also known as CD166 (cluster of differentiation 166), which is a member of a subfamily of immunoglobulin receptors with five immunoglobulin-like domains (VVC2C2C2) in the extracellular domain. **This protein binds to T-cell differentiation antigene CD6, and is implicated in the processes of cell adhesion and migration**. Multiple alternatively spliced transcript variants encoding different isoforms have been found.	Harris & Kerr
CXCR4[17]	This gene encodes a **CXC chemokine receptor specific for stromal cell-derived factor-1**. The protein has 7 transmembrane regions and is located on the cell surface. It acts with the CD4 protein to support HIV entry into cells **and is also highly expressed in breast cancer cells.** Mutations in this gene have been associated with WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome. Alternate transcriptional splice variants, encoding different isoforms, have been characterized.	Harris & Kerr
CD326 (EPCAM[18]**)**	This gene encodes a carcinoma-associated antigen and is a member of a family that includes at least two type I membrane proteins. This antigen is expressed on most normal epithelial cells and gastrointestinal carcinomas and functions as a homotypic calcium-independent cell adhesion molecule. The antigen is being used as a target for immunotherapy treatment of human carcinomas. Mutations in this gene result in congenital tufting enteropathy.	Atlas Baeuerle and Gires
TACSTD2[19]	This intronless gene encodes a carcinoma-associated antigen. This antigen is a cell surface receptor that transduces calcium signals. Mutations of this gene have been associated with gelatinous drop-like corneal dystrophy.	Atlas Hsu et al

[15] https://www.ncbi.nlm.nih.gov/gene/999

[16] https://www.ncbi.nlm.nih.gov/gene/214

[17] https://www.ncbi.nlm.nih.gov/gene/7852

[18] https://www.ncbi.nlm.nih.gov/gene/4072

[19] https://www.ncbi.nlm.nih.gov/gene/4070

Yang et al further note with added simplicity:

Marker	Characteristic Other than as a Putative PCSC Marker
CD133	CD133 is a five-transmembrane domain glycoprotein localizes to membrane protrusions.
CD44	CD44 is a multifunctional surface glycoprotein involved in cell signaling, migration and homing.
CD49b	CD49b plays a critical role in both cell adhesion and lymphocyte activation.
ABCG-2	ABCG-2 contributes to the resistance to chemotherapeutic drugs.
CD24	CD24 is a cell adhesion molecule involved in the regulation of B-cell proliferation and maturation.
CD166	CD166 mediates cell-cell adhesion and also plays a role in development and neutrophil migration.
ALDH-1	ALDH1 is involved in alcohol metabolism and retinoid signaling pathway.

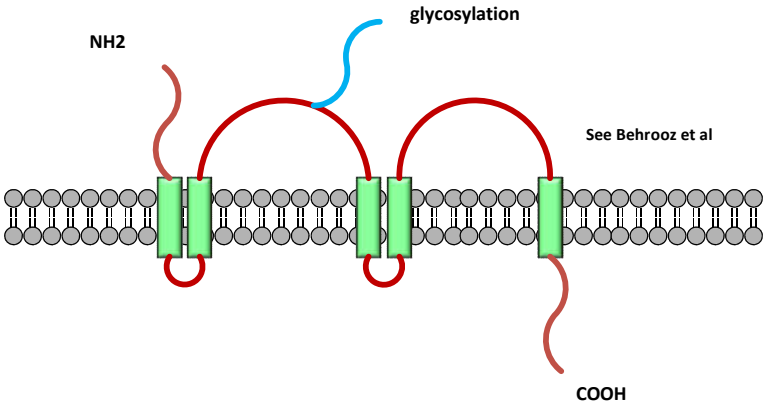
We now consider the key markers in some detail.

3.2 CD133

As Behrooz et al note:

Prominin-1, also known as CD133, contains five transmembrane domains with two large glycosylated extracellular loops and two smaller intracellular loops that comprise 250 and 20 amino acid residues, respectively .

Prominin-1 has a molecular weight of 115/120 kDa and comprises 850–865 amino acids . The N-terminus of prominin-1 is exposed to the extracellular milieu, whereas the C-terminus is exposed to the cytoplasm . The human gene encoding prominin-1 is located on chromosome 4 and contains at least 37 exons. … prominin-1, a plasma membrane cholesterol-binding pentaspan glycoprotein (lipid microdomain), is specifically located to plasma membrane protrusions and accumulates in *membrane lipid microdomains*



They continue:

CD133 (prominin-1), a pentaspan membrane glycoprotein, is one of the most well-characterized biomarkers used for the isolation of cancer stem cells (CSCs).

The presence of CSCs is one of the main causes of tumour reversal and resilience.

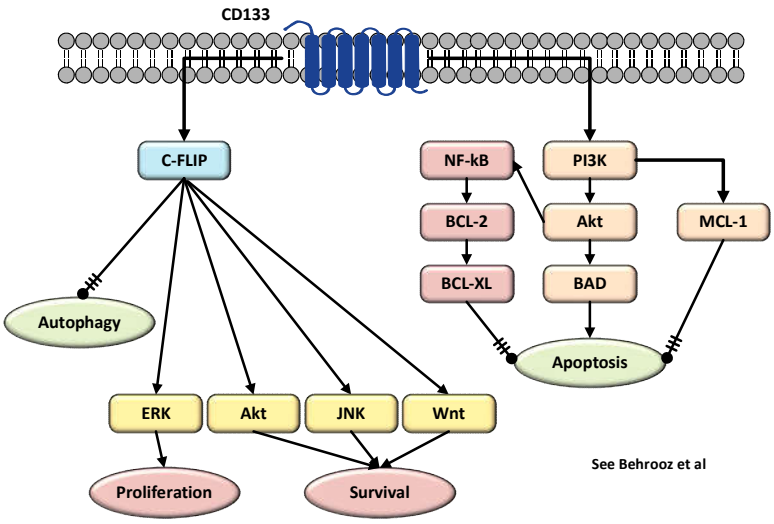
Accumulating evidence has shown that CD133 might be responsible for CSCs tumourigenesis, metastasis and chemoresistance.

It is now understood that CD133 interacts with the Wnt/b-catenin and PI3K-Akt signalling pathways. Moreover, CD133 can upregulate the expression of the FLICE-like inhibitory protein (FLIP) in CD133-positive cells, inhibiting apoptosis.

In addition, CD133 can increase angiogenesis by activating the Wnt signalling pathway and increasing the expression of vascular endothelial growth factor-A (VEGF-A) and interleukin-8.

Therefore, CD133 could be considered to be an 'Achilles' heel' for CSCs, because by inhibiting this protein, the signalling pathways that are involved in cell proliferation will also be inhibited.

By understanding the molecular biology of CD133, we can not only isolate stem cells but can also utilise it as a therapeutic strategy. In this review, we summarise new insights into the fundamental cell biology of CD133 and discuss the involvement of CD133 in metastasis, metabolism, tumourigenesis, drug-resistance, apoptosis and autophagy.



Yang et al note:

The availability of reliable PCSC markers is essential to isolate PCSCs. Just like many other CSCs, the PCSC is likely to share similar antigen expression with PSC, its unmutated counterpart . Accumulated evidence has shown that PCSCs express certain functional and non-functional (phenotypic) markers.

With these markers labelled with antibodies, PCSCs can be identified by flow cytometry (FCM) and isolated by fluorescence-activated cell sorting (FACS) or magnetic cell sorting (MACS). Identification of PCSCs was initially reported by three independent groups in 2005 [4,15,16]. All these initial PCSC research teams have isolated tumorigenic and self-renewing cells from prostate cancer tissues or cell lines with different PCSC markers. The most influential work was from Collins et al. group , who isolated PCSCs from human PC biopsies with CD44+/α2β1high/CD133+ phenotypes.

The isolated PCSCs were capable of differentiation to AR+/PAP+/CK18+ luminal cells. The fundamental evidence to prove the existence of PCSC is the reconstitution of a cancer bulk by inoculation of a small number of cancer cells in a xenograft model. After CD44+/α2β1high/CD133 cells were implanted subcutaneously in mice, formed acini-like structures were found to resemble prostate differentiation .

After that some researchers have utilized a variety of tentative PCSC markers to isolate PCSCs, or PC cells with stemness features, from patient derived tissue or PC cell lines.

These markers include: CD133 , CD44, ABCG-2 , CD24 , CD166 , ALDH1 , integrin α2β1 (CD49b) , Sca-1 , etc.. Most published papers utilized CD133 based combined markers, only a few research teams applied a single marker such as CD133 alone. In addition, the above PCSC markers can be divided into extracellular or intracellular molecules. Extracellular markers technically do not require fixation and permeabilization for antibody binding, so they are more

suitable for isolating living cells, which later will be collected for downstream in vitro or in vivo experiments to analyze PCSC properties.

It is noteworthy that current PCSC markers do not exclusively express on PC, most of them express in CSCs of other cancer types.

3.3 CD 44

As Ouhtit et al note :

CD44, also known as homing cell adhesion molecule is a multi-structural cell molecule involved in cell-cell and cell-extracellular matrix communications.

CD44 regulates a number of central signaling pathways, including PI3K/AKT, Rho GTPases and the Ras-MAPK pathways, but also acts as a growth/arrest sensor, and inhibitor of angiogenesis and invasion, in response to signals from the microenvironment.

The function of CD44 has been very controversial since it acts as both, a suppressor and a promoter of tumor growth and progression.

To address this discrepancy, we have previously established CD44-inducible system both in vitro and in vivo. Next, using microarray analysis, we have identified and validated Survivin, Cortactin and TGF-β2 as novel CD44-downstream target genes, and characterized their signaling pathways underpinning CD44-promoted breast cancer (BC) cell invasion. This report aims to update the literature by adding and discussing the impact of these novel three signaling pathways to better understand the CD44-signaling pathways involved in BC tumor cell invasion….

CD44, a member of the CAM family, along with its role in cellular adhesion, is largely involved in intracellular signaling for cell growth, proliferation and motility . CD44 is involved in the onset of several tumors including neuroblastomas, breast, ovarian, cervical, prostate, lung and, colon cancers . While in neuroblastomas, CD44 acts as a tumor suppressor, its role is controversial in prostate (PC) and breast cancers (BC). Interestingly, in BC, CD44 expression was associated with poor and favorable outcomes, thus indicating the dual nature of CD44 in mediating breast tumor development.

Therefore, in order to address this discrepancy, and further elucidate the mechanisms by which CD44 acts to either suppress or promote BC invasion, our laboratory established a tetracycline inducible systems both in vitro and in vivo, and demonstrated that induction of CD44 promoted breast tumor cell invasion in vitro , as well as metastasis of breast tumor to the liver.

Chen et al note:

One hundred forty-eight prostate tissues composed of prostate cancer (PCa), high-grade prostatic intraepithelial neoplasia (HGPIN), and benign prostate hyperplasia (BPH) were immunostained for CD44 and CD133. A higher level of CD44 expression was observed in 42%

of PCa, 57% of HGPIN, and 42% BPH tissues, suggesting that CD44 expression was not correlated to malignant stage of prostate cancer .

The roles of CD44 isoforms have also been investigated in prostate cancer. The expression levels of CD44s and all its 9 variants were analyzed in 94 human surgical specimens, whereas the control group consisted of 14 specimens from patients with benign prostatic hyperplasia. In localized prostate cancer, CD44s was underexpressed and all the other isoforms were overexpressed. A higher expression of CD44v2 independently correlated with a better recurrence-free survival rate .

RNAseq was used to determine the differential expression of membrane proteins on PC3 cells that possessed epithelial or mesenchymal phenotypes. PC3-epithelial cells had a higher level of CD44v6 as confirmed by flow cytometry and Western blot analysis . The functional role of CD44v6 in prostate cancer cells was assessed using a small interfering RNA (siRNA) approach.

Knock-down of CD44v6 caused a loss of EMT markers and reduced tumorigenic potential, tumor sphere formation, and enhanced chemo/radiosensitivity . A separate study showed that silencing CD44v9 using short hairpin RNA inhibited assembly of p-Met, AR, HSP90, P110α/PI3K, and CD44 into lipid raft-like structures and reversed the assembly of these components in the complex in prostate cancer. HGF-induced activation of HGF-receptor/c-Met and stimulated hyaluronan/CD44v9 signaling transduction. This, in turn, stabilized the androgen receptor functions in prostate cancer cells .

The roles of miRNAs on CD44 expression have been examined in PCa models. miR-34a, miR-106a, miR-141, and let-7b were downregulated on stem/progenitor cells which expressed CD44. miR-301 and miR-452 were overexpressed on CD44-positive cells . CD44 is predicted as a direct target of miR-199a-3p using target prediction program and this was partially confirmed using a luciferase reporter assay in PCa cells . miR- 383 expression repressed CD44 mRNA and protein expression in DU145/PC3/LNCaP cells . These studies indicate that miRNAs play a role in regulating CD44 expression in PCa.

As Iczkowski et al have noted:

Prostate cancer (PC) consistently overexpresses variant the (v) isoform of the cell adhesion protein CD44, and loses expression of the standard (s) isoform. ... We re-expressed CD44 full-length (exons 1-20) or standard (exons 1-5 + 16-20) or enforced stable RNAi against CD44v, and the examined functional effects on PC. The effect of stable knockout of calcitonin, a paracrine factor, or its receptor, on CD44 was assessed.

...Re-expression of full-length CD44 or CD44s increased the total CD44 mRNA and CD44s protein while suppressing CD44v. These approaches, and RNAi to CD44v, decreased invasion. In adhesion assays, benign prostate cells bound mainly to hyaluronan, whereas PC lost affinity for hyaluronan but bound more strongly to fibronectin.

Re-expressing CD44s restored predominant hyaluronan binding. Knockout of the calcitonin receptor in PC-3 derived cells caused marked loss of CD44v expression and reversion to CD44s

expression. … Calcitonin influenced PC's balance between CD44s and CD44v. CD44v controlled invasiveness, altered ligand binding, and provides a target for therapeutic intervention

Xu et al note:

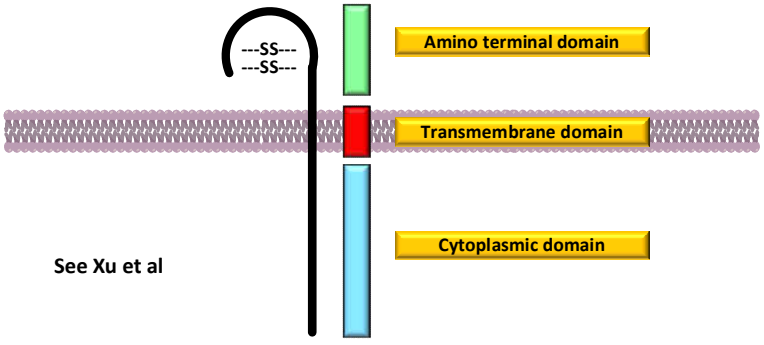
CD44 protein consists of a short C-terminal cytoplasmic domain, a transmembrane domain, and seven extracellular domains which contain an N-terminal HA-binding link-homology module and stem region.in CD44v, variant exons are inserted into the stem region at the proximal plasma membrane external region.

We show below the protein character at the cell surface. The authors continue:

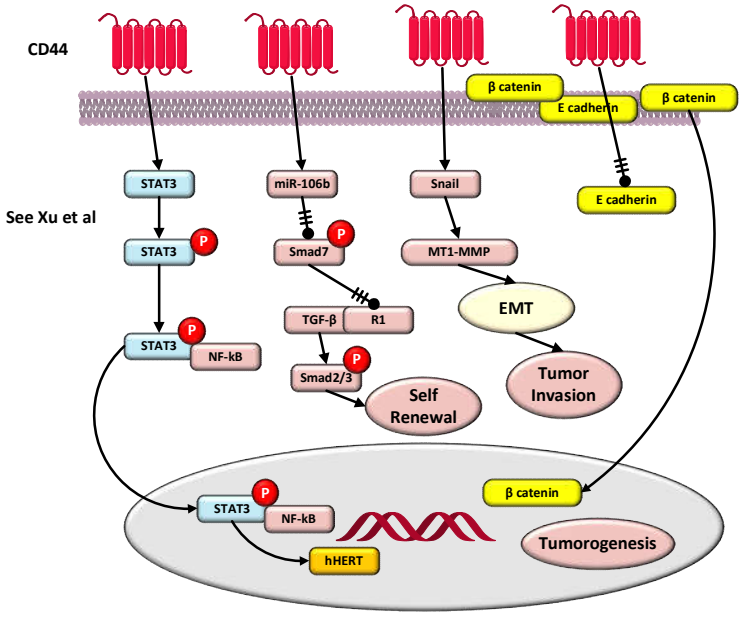
CD44, a complex transmembrane glycoprotein, also called Hermes antigen, homing cell adhesion molecule, HUTCH-1, phagocytic glycoprotein-1, lymphocyte-homing receptor, and ECM-III, is encoded by the CD44 gene on chromosome 11,1 which consists of 20 exons.2 Transcripts for the CD44 gene undergo complex alternative splicing, which results in many functionally distinct isoforms, such as CD44 standard isoform (CD44s) and CD44 variant isoform (CD44v).3

The smallest CD44s is encoded by constant exons 1–5 and 16–20 and translated into a polypeptide of a molecular mass of 80–85 kDa (Figure 1).4 Exon 1 is an N-terminal signal sequence, exons 2 and 3 are a link module that binds to hyaluronic acid (HA), exons 4, 5, 16, and 17 compose a stem region, exon 18 makes up a single-pass transmembrane domain, and exon 20 forms a cytoplasmic domain. Exon 19 is spliced out in all forms of CD44 cDNAs.4 Alternative splicing is the basis not only for the structural but also for functional diversity of this protein. Multiple CD44v is produced by insertion of variant exons (v1–v10) at the proximal plasma membrane external region.

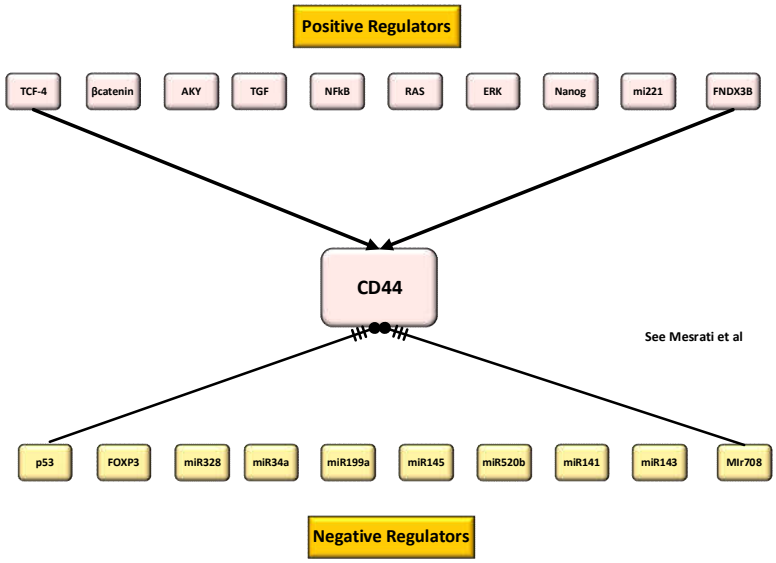
CD44s is found in most cells,5 while CD44v is expressed primarily on cells during inflammation and on tumor cells.6–8 CD44 protein consists of a short C-terminal cytoplasmic domain, a transmembrane domain, and seven extracellular domains which contains an N-terminal HA-binding link-homology module and stem region



Now Xu et al provide a basis for the pathways that CD44 activates as shown below:



Mesrati et al presents the following Figure depicting the positive and negative regulators.



3.4 PSMA

PSMA is also known as the Prostate Specific Membrane Antigen, is a surface protein which is highly expressed on prostate cells, especially those that are androgen resistant and metastatic. It has been used as a target for PET scans and somewhat for prognostic evaluation. The effects of PSMA have been understood to some degree whereas its expression control does not yet seem to

be fully understood. PSMA is both correlative and causative of PCa metastatic growth. It also presents an interesting cell target for a variety of therapeutic strategies.

3.4.1 Gene and Protein

We begin with the NCBI definition which notes[20]:

This gene encodes a type II transmembrane glycoprotein belonging to the M28 peptidase family. The protein acts as a glutamate carboxypeptidase on different alternative substrates, including the nutrient folate and the neuropeptide N-acetyl-l-aspartyl-l-glutamate and is expressed in a number of tissues such as prostate, central and peripheral nervous system and kidney.

A mutation in this gene may be associated with impaired intestinal absorption of dietary folates, resulting in low blood folate levels and consequent hyperhomocysteinemia. Expression of this protein in the brain may be involved in a number of pathological conditions associated with glutamate excitotoxicity.

In the prostate the protein is up-regulated in cancerous cells and is used as an effective diagnostic and prognostic indicator of prostate cancer. This gene likely arose from a duplication event of a nearby chromosomal region. Alternative splicing gives rise to multiple transcript variants encoding several different isoforms.

We now present a summary of what is understood about PSMA. As Caromile et al note:

*PSMA is a **750–amino acid type II transmembrane peptidase enzyme** that is encoded by the folate hydrolase 1 (FOLH1) gene. Although PSMA is also known as glutamate carboxypeptidase II, N-acetyl-Laspartyl-L-glutamate peptidase I, and N-acetylaspartylglutamate peptidase, those studying PCa or general oncology commonly use the term PSMA, which will be used here.*

It has been shown that PSMA is present in low amounts on prostate epithelial cells and is progressively up-regulated during disease progression in prostate tumors, in which it correlates negatively with prognosis and consequently may be a promising tool for the diagnosis, detection, localization, and treatment of PCa.

Currently, PSMA is used as an immunoscintigraphic target in the clinic to direct therapy to androgen-independent prostate tumors. RNA aptamers selectively targeting PSMA enzymatic activity have also been successful in slowing primary tumor growth in murine models.

Although we have previously shown that endothelial-expressed PSMA regulates angiogenesis and retinal neovascularization primarily via b1 integrin–mediated cell adhesion, an important functional role for PSMA in PCa has not been demonstrated.

Caromile et al continue:

[20] https://www.ncbi.nlm.nih.gov/gene/2346

….directly underlies prostate tumor progression in vivo. We found that tumors in wild-type animals were larger and of higher grade with a higher microvessel density as compared to tumors in the PSMA knockout animals, which is consistent with our previous results implicating PSMA as an angiogenic regulator.

In addition, PSMA-positive tumor cells were viable at greater distances from the vasculature than their PSMA knockout counterparts, suggesting that cell-intrinsic survival components also contribute to tumor growth.

Accordingly, wild-type tumors expressed relatively greater amounts of IGF-1R and exhibited greater activation of the phosphatidylinositol 3-kinase (PI3K)–AKT pathway, whereas tumors lacking PSMA not only had decreased IGF-1R expression but also had diverted signaling downstream of PI3K-AKT to the mitogen-activated protein kinase (MAPK)–extracellular signal–regulated kinases 1 and 2 (ERK1/2) pathway, consistent with a PSMA-dependent signaling switch.

Moreover, manipulation of PSMA expression in mouse TRAMP-C1 cell lines and human PCa cell lines recapitulated this change in signaling. Analysis of publicly available gene expression data sets from PCa samples confirmed that high PSMA expression was predictive of a high Gleason score.

In addition, patient samples with high PSMA expression and high Gleason scores displayed a prosurvival gene expression signature with increased expression of the antiapoptotic marker survivin and IGF-1R, consistent with a role for PSMA in the regulation of signal transduction in human PCa disease as well. Therefore, in addition to its role as a PCa marker and target, our results indicate that increasing amounts of PSMA in prostate tumor epithelium serve to drive prosurvival mechanisms and thus identify it as a functional regulator of prostate tumor progression. These findings also suggest that PSMA-positive tumors may be more sensitive to PI3K pathway inhibitors and less sensitive to MAPK pathway inhibitors.

3.4.2 Functions

What function does PSMA play? Science Signalling notes Conway et al who observe:

Prostate-specific membrane antigen (PSMA) is so-named because its expression is enhanced in advanced prostate carcinomas, where its increased presence correlates with a poor prognosis. The protein is also called glutamate carboxypeptidase II and is a transmembrane protein with peptidase activity.

***PSMA has been found in endothelial cells in tumor vasculature. Given roles of other peptidases in angiogenesis**, Conway et al. explored the possibility of such a role for PSMA. They used an in vivo angiogenesis assay in knockout mice lacking PSMA to show that loss of the PSMA protein inhibited formation of new blood vessels.*

Proteolysis contributes to remodeling of the extracellular matrix that is necessary for angiogenesis, but further studies by the authors suggest that PSMA may instead be part of a

complex regulatory loop that controls integrin signaling and activation of the p21-activated kinase 1 (PAK1). In vitro cell invasion studies with PSMA-null cells or with inhibitors of the enzyme showed that PSMA has an important role in cell invasion and in signaling from β1 integrins to focal adhesion kinase (FAK) and PAK1.

The authors confirmed that PSMA interacts with the actin-binding protein filamin A. Disruption of this interaction with a peptide designed to compete with PMSA for binding to filamin A decreased the peptidase activity of PMSA and decreased phosphorylation of PAK1 in cultured cells. PAK1 also interacts with filamin A, and the authors propose that it may compete with PMSA for binding to filamin A.

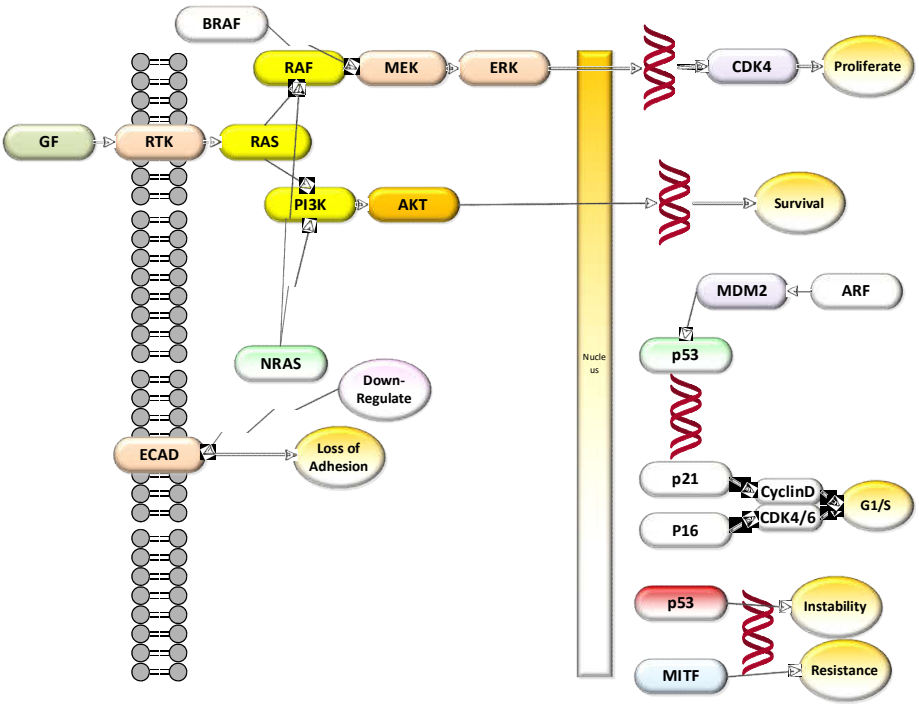
The interaction of PMSA and the cytoskeletal protein filamin A may allow a feedback signal from integrin β1 and PAK to keep PMSA activity in check. Inhibition of PAK by expression of a peptide corresponding to its autoinhibitory domain enhanced association of PMSA with filamin A, increasing its peptidase activity. Further understanding of PMSA's roles in control of angiogenesis may allow new strategies to inhibit angiogenesis in cancers and other diseases to which it contributes.

Thus PSMA can become a significant driver of a multiplicity of downstream proteins and genes.

3.4.3 Downstream Pathways

PSMA is also known as the Prostate Specific Membrane Antigen. It is a putative target for attacking malignant prostate cancer cells. There has been recent interest in this transmembrane protein as a target for various imaging modalities. Moreover, it has an interest as a target for a multiplicity of therapeutic modalities. We examine this marker as a means for several of these therapeutic modalities. The objective is to consider how we can “engineer” a therapeutic strategy using the many tools now available.

One of the targets for PSMA is AKT. We show below a generic flow on actions with AKT at a central role.



As Kaittanis et al have noted:

Prostate-specific membrane antigen (PSMA) or folate hydrolase 1 (FoLH1) is highly expressed on prostate cancer. Its expression correlates inversely with survival and increases with tumor grade. However, the biological role of PSMA has not been explored, and its role in prostate cancer remained elusive. Filling this gap, we demonstrate that in prostate cancer,

PSMA initiates signaling upstream of PI3K through G protein–coupled receptors, specifically via the metabotropic glutamate receptor (mGlur)*.*

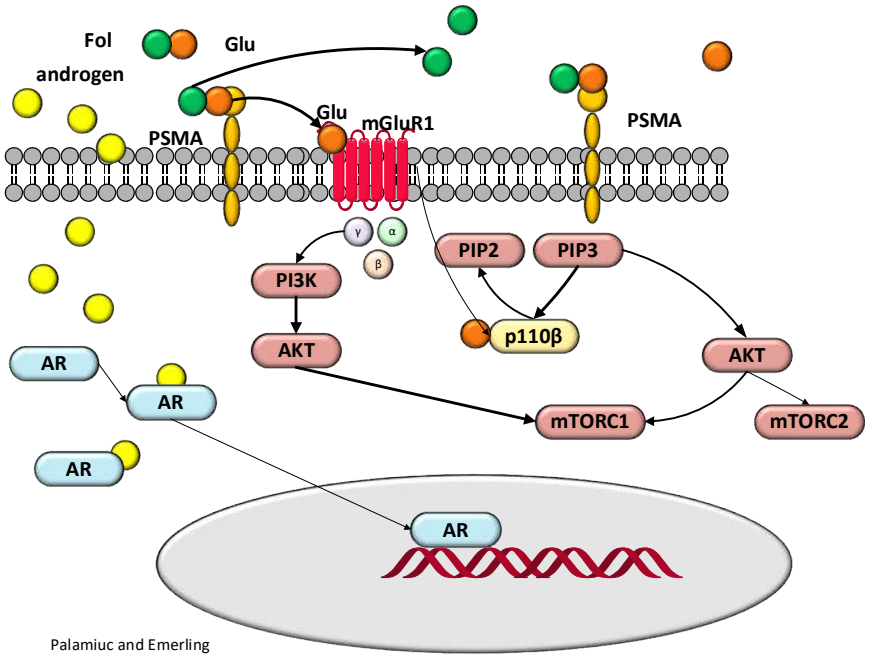
PSMA's carboxypeptidase activity releases glutamate from vitamin B9 and other glutamated substrates, which activate mGlur I. Activated mGlur I subsequently induces activation of phosphoinositide 3-kinase (PI3K) through phosphorylation of p110β independent of PtEn loss. the p110β isoform of PI3K plays a particularly important role in the pathogenesis of prostate cancer, but the origin of its activation was so far unknown.

PSMA expression correlated with PI3K–Akt signaling in cells, animal models, and patients. We interrogated the activity of the PSMA–PI3K axis through positron emission tomography and magnetic resonance imaging. Inhibition of PSMA in preclinical models inhibited PI3K signaling and promoted tumor regression. our data present a novel oncogenic signaling role of PSMA that can be exploited for therapy *and interrogated with imaging*

What is attractive in this case is that there appears in PSMA to be a cell surface marker targetable in malignant cells. We examine this marker in some detail and then examine ways in which it can be utilized in a therapeutic manner. For example, we can use PSMA as an epitope for immunotherapeutic attack. We could also use it as a target for viral insertion. Thirdly we could

use bi-specific antibodies for the delivery of cancer attacking therapeutics, in short it becomes a useful target for a variety of therapeutic application.

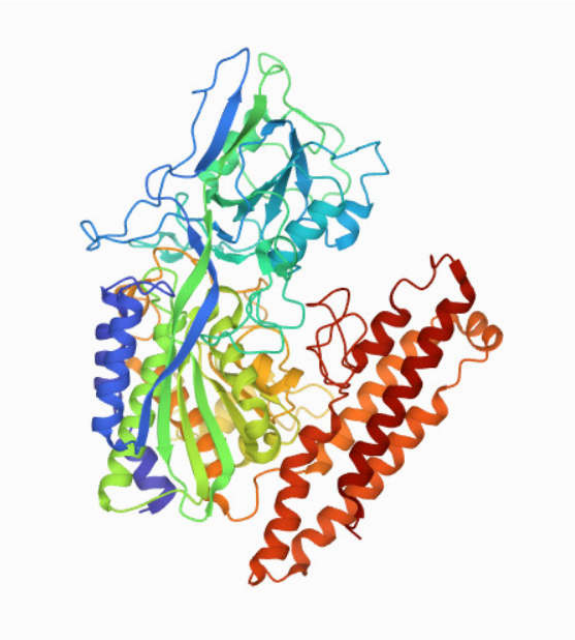
The authors, Palamiuc and Emrling, also note the dynamics using the PSMA work of Kaittanis et al, as follows:



Note the PSMA releases glutamate from the bound form and this glutamate then binds to the receptor and activates Akt. We shall examine this in detail in the next section. Now PSMA protein[21] containing 695 nucleic acids is shown below.

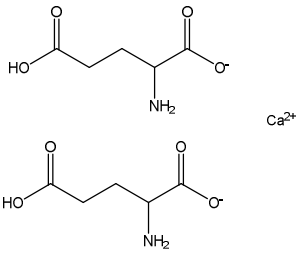
Details of the PSMA protein structure are shown in the following.

[21] https://www.rcsb.org/structure/1Z8L

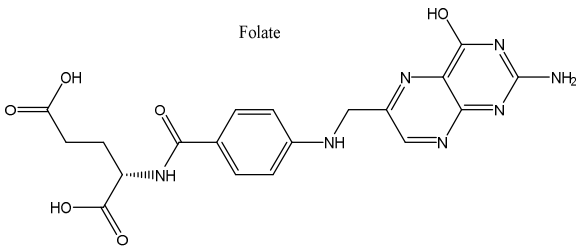


We also provide below the chemical forms of Glu and Fol as are effective in this model.

Glutamate



Folate



3.4.4 Immuno Targeting

As Dang et al note :

In CaP, tumor cells express a number of prostate-specific surface proteins that represent promising targets for therapy including PSMA, a type II transmembrane protein expressed predominantly on prostate cells. PSMA is an attractive target due to its low expression on non-

prostatic tissue and its overexpression in a majority of CaP tumors, with expression level correlated to tumor stage and aggressiveness.

The overexpression of PSMA in CaP has been shown to promote tumor progression through the aberrant activation of PI3K-AKT signaling pathways.

Therefore, a diverse array of PSMA-targeted therapies are in development including radionuclides, antibody drug conjugates, chimeric antigen receptor T cells and T cell engagers (TCEs) TCEs are heterodimeric antibodies engineered to simultaneously bind to a tumor-associated antigen on cancer cells and to CD3 on T cells, forming an immunological synapse that promotes T cell redirected lysis of tumor cells in an MHC-independent manner.

Although T cell redirection is promising, all such approaches to date induce strong pan-T cell stimulation and toxic immune activation culminating in the systemic release of proinflammatory cytokines leading to cytokine release syndrome (CRS). This major side effect of TCE therapy can potentially be attributed to the fact that a majority of TCEs developed thus far incorporate strong binding/activating anti-CD3 moieties with affinities in the 1–200nM range such as OKT3 and UCHT1. Despite their effectiveness in hematologic tumors, TCEs have also demonstrated limited success in solid tumors, and combination treatments may be needed to maximize efficacy. Currently, a number of TCEs targeting PSMA and CD3 are being investigated in early phase clinical studies for the treatment of mCRPC.

Pasotuxizumab (AMG 212), a PSMA-targeting bispecific T cell engager (BiTE), demonstrated signs of clinical activity in a phase I study as approximately one-third of patients in the 20, 40, and 80μg/dose groups of the continuous intravenous infusion (cIV) cohort exhibited a >50% decline in Prostatespecific antigen (PSA) levels.20 Due to a sponsor change, this study was terminated early, and the maximum tolerated dose for the cIV cohort was not determined.

AMG 160 is a half-life extended BiTE also being investigated in phase I clinical trials. In contrast to the high-frequency, once-daily dosing regimen of pasotuxizumab, AMG 160 is administered once every 2weeks.

Data from the phase I study indicate that 27.6% of patients had a confirmed PSA response to AMG 160, although grade 2 and grade 3 CRS occurred in 60.5% and 25.6% of patients, respectively. For HPN424, a once-weekly dosed TriTAC molecule targeting PSMA and CD3, clinical activity at the highest fixed dose tested (160ng/kg) was demonstrated by PSA reduction in three out of seven patients with one confirmed partial response.

Overall, these phase I clinical trial data suggest that, although promising, there are shortcomings associated with current PSMA-targeted TCE therapeutics, including either safety (polycytokine secretion and clinical CRS), efficacy, or dosing schedule, that could be addressed by an IgG TCE that facilitates tumor killing with minimal cytokine release.

3.5 CD326 (EPCAM)

As Ni et al note[22] :

*Despite significant advances in surgery, radiotherapy and chemotherapy to treat prostate cancer (CaP), many patients die of secondary disease (metastases). Current therapeutic approaches are limited, and there is no cure for metastatic castration-resistant prostate cancer (CRPC). Epithelial cell adhesion molecule (**EpCAM, also known as CD326**) is a transmembrane glycoprotein that is highly expressed in rapidly proliferating carcinomas and plays an important role in the prevention of cell-cell adhesion, cell signalling, migration, proliferation and differentiation.*

Stably and highly expressed EpCAM has been found in primary CaP tissues, effusions and CaP metastases, making it an ideal candidate of tumour-associated antigen to detect metastasis of CaP cells in the circulation as well as a promising therapeutic target to control metastatic CRPC disease. In this review, we discuss the implications of the newly identified roles of EpCAM in terms of its diagnostic and metastatic relevance to CaP. We also summarize EpCAM expression in human CaP and EpCAM-mediated signalling pathways in cancer metastasis. Finally, emerging and innovative approaches to the management of the disease and expanding potential therapeutic applications of EpCAM for targeted strategies in future CaP therapy will be explored.

3.6 TACSTD2

As Hsu et al note :

Tumor-associated calcium signal transducer 2 (Tacstd2, Trop2, TROP2) is a cell surface glycoprotein that has emerged as a promising therapeutic target due to its overexpression in multiple epithelial cancers.

Sacituzumab govitecan (IMMU-132), an anti-Trop2 antibody conjugated with SN-38, a cytotoxic agent that targets DNA replication, has shown therapeutic activity in several malignances, including triple negative breast cancer, advanced non-small cell lung cancer, and metastatic platinum-resistant urothelial carcinoma (10, 12–14).

In the prostate, Trop2 is an important regulator of prostate stem cell self-renewal.

Murine prostate basal cells with high levels of Trop2 can regenerate prostatic tubules, and Trop2-positive epithelial cells are enriched after androgen ablation , suggesting that Trop2 has roles in cell survival after androgen ablation and lineage plasticity during the regeneration of prostate-like structures after androgen repletion. Trop2 is activated through proteolytic cleavages (15, 17) and is involved in prostate cancer metastasis through β1 integrin and FAK signaling (18, 19).

Herein, we demonstrate that an elevated level of Trop2 is prognostic for biochemical recurrence and even higher level of Trop2 is found in metastatic CRPC and NEPC. Deletion of the TROP2

[22] https://pubmed.ncbi.nlm.nih.gov/22718399/

gene significantly slows cell growth and decreases migration, invasion, and metastatic colonization of prostate cancer cells while overexpression of Trop2 increases growth, invasion, and metastasis.

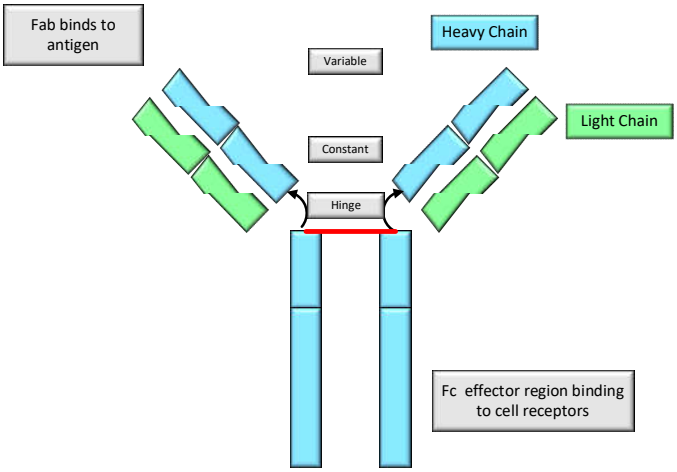
Overexpression of Trop2 leads to a significant decrease in luminal markers, such as androgen receptor (AR), and Trop2 enhances tumor growth while also inducing lineage plasticity, neuroendocrine features, and metastasis in vivo.

Trop2- expressing prostate cancer xenografts are resistant to androgen ablation and express high levels of PARP1. PARP1 inhibitors decrease cell proliferation and neuroendocrine markers and suppress the growth and metastasis of Trop2-driven tumors.

4 ANTIBODY OPTIONS

The use of antibodies in cancer therapeutics has seen great advancements in the past twenty years. From simple targeted Ab such as Herceptin to more complex one delivering chemotherapeutic agents to targeted cells. The recent article by Jin et al is an excellent summary of these efforts. We will review some of these current options and suggest some additional ones.

The fundamental Ab structure is the IgG paradigm which we show below:



It is this paradigm that we can modify in a variety of ways. It can be customized and reproduced extensively. It then presents a highly flexible platform for delivering specific therapeutics to targeted Ag on cancer cells.

Antibodies are a powerful tool in the attack on cancers. As Hansel et al note :

Among the advantages of protein therapeutics such as mAbs over conventional low-molecular-mass drugs are their high specificities, which facilitates precise action, and their long half-lives, which allows infrequent dosing.

Furthermore, molecular engineering technologies have enabled the structure of mAbs to be fine-tuned for specific therapeutic actions and to minimize immunogenicity, thus improving their risk–benefit ratio. This is reflected in mAbs having approval rates of around 20% compared with 5% for new chemical entities. However, in addition to a range of adverse events that may be generally associated with therapeutic mAbs, there are also adverse effects that are related to the specific target or mechanism of action…

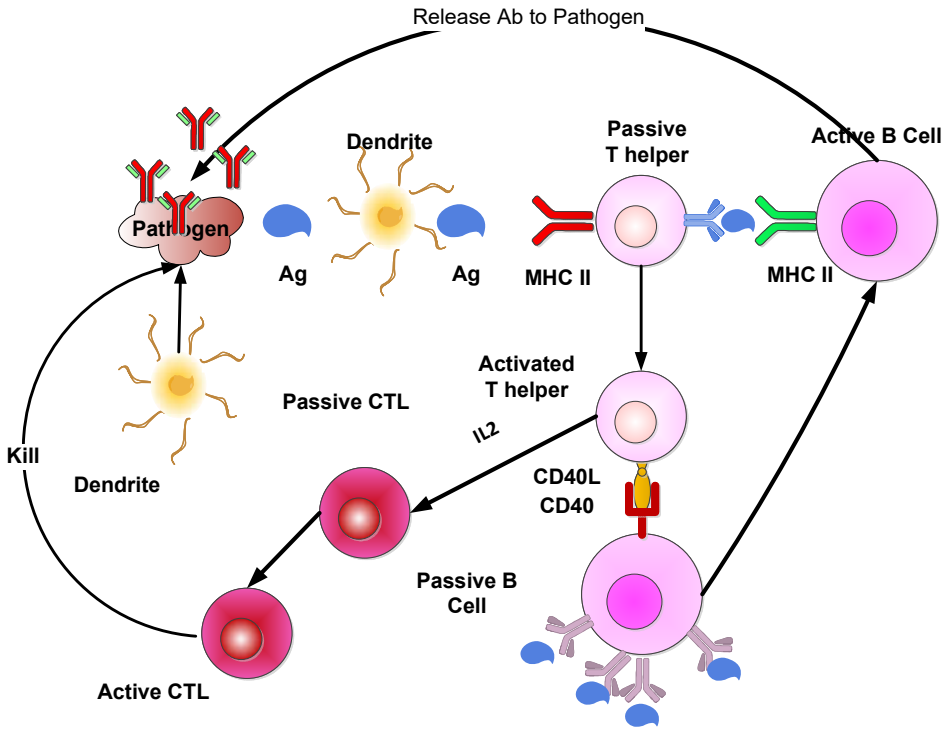
Antibodies operate through various mechanisms.

When the Fab part of an antibody binds to the antigen it blocks its interaction with a ligand. Signalling occurs when the binding of the antibody to a receptor delivers an agonist signal. These functions can be independent of the Fc part of the molecule (although interactions of the Fc portion with other molecules can enhance these mechanisms). In addition, the antibody can

exert actions through its Fc region: these include antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity and antibody-dependent cellular phagocytosis. Furthermore, the constant heavychain domain regions (CH2 and CH3) of Fc on immunoglobulin G (IgG) interact with the neonatal Fc receptor (FcR) to influence transport of IgG across cellular barriers and regulate the circulating levels of the antibody thus, extending its half-life. Recruitment of these effectors is dependent on the isotype of the antibody, and its ability to recruit complement or effector cells. IgG1 is the most commonly used subclass of Ig to trigger cell death.

4.1 IMMUNE SYSTEM ARCHITECTURE

Let us return to the overall immune system architecture. The Figure below depicts the complex nature of a Target cell, pathogen, being recognized and attacked. The antibody element is a result of a recognition of some antigen on the Target and the B cells being activated via various mechanisms and then the B cell having a matching Ab being activated to produce those Abs en masse. The result is an explosion of specific Abs and their dissemination throughout the body, their attaching themselves to the Target cells and the activation of the Complement system, the proteins generated in the liver and freely flowing in the blood stream, to neutralize the Target cells.

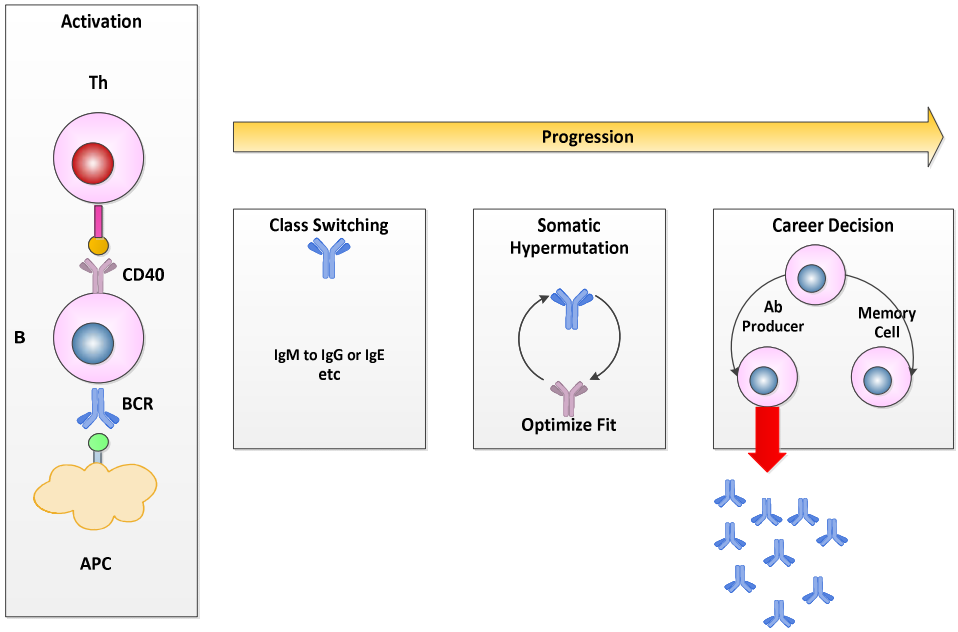


Recall that interaction of antibody with antigen initiates the classical pathway of complement activation. This biochemical cascade of enzymes and protein fragments facilitates destruction of microbes by the membrane attack complex (MAC), by increased opsonization through C3b binding of microbial surfaces and by the production of anaphylatoxins C3a, C5a, and C4a.

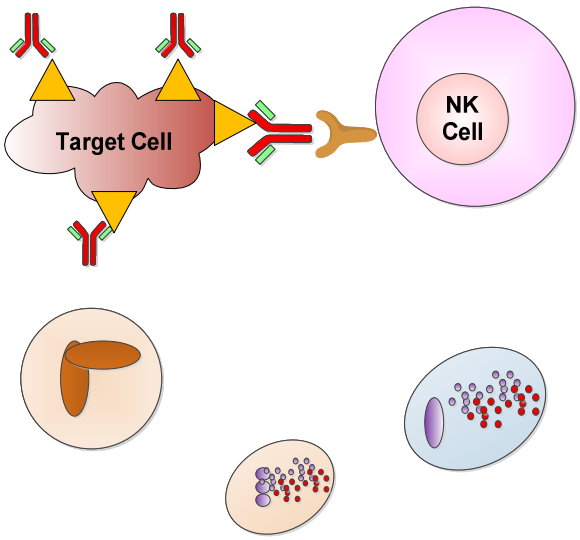
The cascade begins with the activation of component C1. Binding of IgM or IgG antibody to antigen causes a conformational change in the Fc region of the immunoglobulin molecule. This conformational change enables binding of the first component of the classic pathway, C1q. Each head of C1q may bind to a Ch2 domain (within the Fc portion) of an antibody molecule.

Upon binding to antibody, C1q undergoes a conformational change that leads to the sequential binding and activation of the serine proteases C1r and CIs. The C1qrs complex has enzymatic activity for both C4 and C2, indicated by a horizontal bar as either C1qrs or abbreviated as C15. Activation of C1qrs leads to the rapid cleavage and activation of components C4, C2, and C3. In fact, both the classical and mannan-binding lectin (MBL) pathways of complement activation are identical in the cleavage and activation of C4, C2, and C3

The Ab process is detailed more closely below. Note that some activated B cells produce Abs while others are held in abeyance for another future attack.



The above Figure depicts the process of Ab generation. The issue at hand is; what happens with the Ab and what kills off these bad cells? That is particularly important in understanding how to deal with cancer. Cells are eliminated via the interaction of phagocytes as well as the Complement system, part of the innate immune system. As we have noted earlier the NK cells can use the Abs as an indicator of targeting. We show this below along with some of the other phagocytes such as macrophages and neutrophils.



The Complement System is what attacks the Target Cell when it is covered with Abs. As Merle et al note:

Complement is a central part of the innate immunity that serves as a first line of defense against foreign and altered host cells. The complement system is composed of plasma proteins produced mainly by the liver or membrane proteins expressed on cell surface. Complement operates in plasma, in tissues, or within cells. Complement proteins collaborate as a cascade to opsonize pathogens and induce a series of inflammatory responses helping immune cells to fight infection and maintain homeostasis.

The complement system can be initiated depending on the context by three distinct pathways – classical (CP), lectin (LP), and alternative (AP), each leading to a common terminal pathway. In a healthy individual, the AP is permanently active at low levels to survey for presence of pathogens.

Healthy host cells are protected against complement attack and are resistant to persistent low-grade activation. The three pathways are activated on the surface of apoptotic cells, which are constantly generated within the body during normal cellular homeostasis This complement activation is tightly regulated to eliminate dying cells without further activation of other innate or adaptive immune components. Complement is only fully activated in cases of pathogen infection. During an infection, complement leads to inflammation, opsonization, phagocytosis, and destruction of the pathogen and ultimately results in activation of the adaptive immune response. Both inefficient and over stimulation of complement can be detrimental for the host and are associated with increased susceptibility to infections or non-infectious diseases, including autoimmunity, chronic inflammation, thrombotic microangiopathy, graft rejection, and cancer.

The antibody-dependent cell-mediated cytotoxicity can be described as follows. The “tagging" of an invasive organism can attract phagocytic cells and other cytolytic cells. FcRs on NK cells (FcyRIII) and eosinophils (FcyRI, FcbRI, and FcotRI) are IgG-, IgE-, and IgA-specific. The bound cells may be bacteria, protozoa, or even some parasitic worms. As with phagocytic cells,

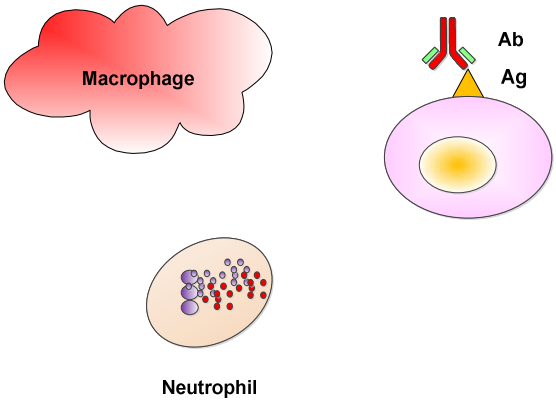
these receptors allow the cytolytic cells to bind invasive organisms “tagged" with IgG, IgE, or IgA antibodies, but rather than engulfment, they use cytolytic mechanisms to kill the “tagged" organisms. This process is termed antibody-dependent cell-mediated cytotoxicity (ADCC). The cytolytic mechanisms used by NK cells and eosinophils in ADCC are similar to some of those used by cytotoxic T cells to kill the intruder.

The Complement activation can proceed as follows. The classical pathway of complement is activated by conformational changes that occur in the Fc portion of antibodies upon epitope binding. Antibodies (usually of the IgM and IgG isotypes) facilitate the sequential binding of the C1, C4, C2, and C3 components of the complement system. Like the alternative and mannan-binding lectin pathways, completion of the classical complement pathway results in the production of C3b, a “sticky"

As noted by Merle et al (II):

The main role of complement in pathogen elimination is indirect, namely, the deposition of complement fragments on the surface of pathogen targets, so-called opsonization that allows their recognition, ingestion, and destruction by phagocytic cells, neutrophils, monocytes, and macrophages. Both IgG antibodies and C3 fragments are the classical opsonins. But complement opsonization, resulting from the direct activation of the AP on pathogens surface allows their elimination by phagocytes before the mounting of a response and the appearance of antibodies.

We demonstrate some of these effects below.



Thus, the process is somewhat simple:

1. Target cells produce an antigen

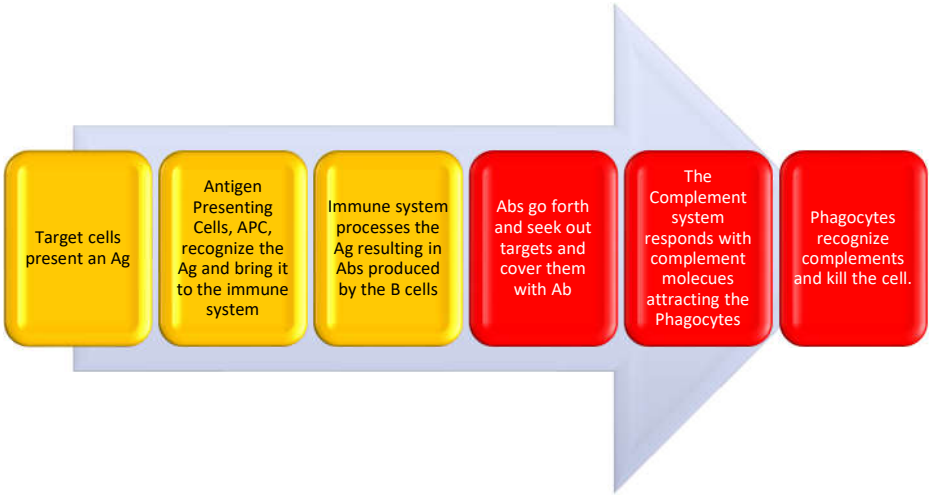
2. Antigen presenting cells see the Ag and carry it to the adaptive system.

3. B cells are activated by the antigen and they produce Abs targeted to the Ag

4. The Abs go out and cover the target cells

5. The Abs attract the Complement system proteins which cover the target as well

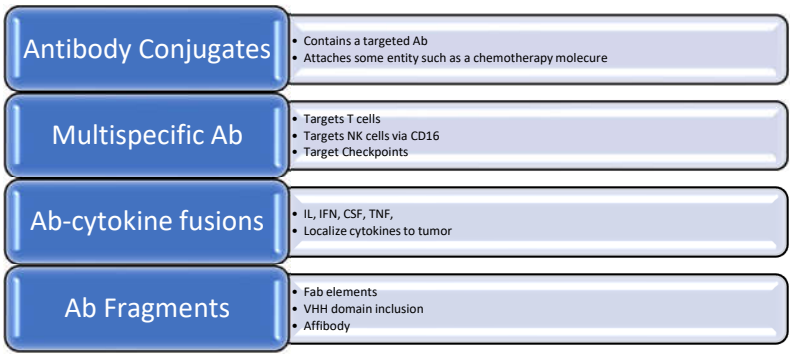
6. The phagocytes are brought out to kill off the complement targeted cells.



Thus, we see this as an orchestrated process between the elements of the immune system all playing parts in seeking out and destroying invaders. Protection of "self" is a key part of this rather aggressive process and that we leave to the well-established literature.

4.2 APPROACHES

There are multiple approaches to utilizing Abs. We show graphically some below and discuss them in detail latter. All require a targeting Ag to attach to the cell in question.



We will examine several of these options. Jin et al have examined these in great detail.

4.3 BI-SPECIFICS

We can now move on to bi-specific antibodies. Bi-specific antibodies have recently become more readily available and can perform multiple therapeutic effects simultaneously.

As Kaiser has noted regarding some historical elements:

Bispecific antibodies offer a third way to harness T cells. In the mid-1980s, cancer researchers began to engineer antibodies that had two tips—one matched to a cancer cell antigen and the other to a T cell surface protein called CD3. The idea was to directly link T cells to tumor cells, thereby skipping the need for T cells to learn to attack a cancer. "It's mimicking what naturally happens, but the advantage is that you can engage all T cells," not just those trained to attack the tumor, says Dirk Nagorsen, a vice president and cancer researcher at Amgen.

In 1985, the field was galvanized by two reports in Nature that such a "bispecific" antibody could destroy cancer cells in a dish; studies soon showed those antibodies could shrink tumors in mice. The drugs were hard to make. Antibodies are modular, with two identical "heavy" chains, making up the stem and half of each arm of the Y, and two identical "light" chains, each of which completes one arm. Trying to assemble bispecific antibodies from those complex components, protein chemists got 10 versions of each molecule.

That outcome meant laborious efforts to sift out the one researchers wanted The first bispecific antibody for cancer was approved in Europe in 2009. It was meant to mop up the malignant cells that cause abdominal fluid to build up in some cancer patients—but it didn't work that well, so the drug only stayed on the market a few years.

The field regained momentum, however, after Amgen snapped up Micromet in 2012 and later showed that its BiTE drug, blinatumomab (Blincyto), doubled the survival time of patients with advanced acute lymphocytic leukemia. Beginning in 2014, the Food and Drug Administration approved the drug to treat several adult and pediatric forms of the disease. Amgen is now testing BiTEs for other cancers, including myeloma and lung, prostate, and brain cancers. …

Solid tumors are a challenging target for bispecifics in part because tumors often lack a unique antigen for the antibodies to grab. Many tumors are also surrounded by blood vessels, tissue, and immune cells that form a barrier T cells can't easily penetrate.

The issue with solid tumors is critical. The most important part of a MaAb functioning is the Ag target. To be effective the target must be singular to the target and thus not one on a multiplicity of other cells. Furthermore for solid tumor we must be able to reach the cells. This is often the most difficult part. If the drug is administered in some IV manner we then must know that the targets are adequately perfused and that there can be a ready extravasation from the blood stream to the cells. Furthermore we need to have adequate supplies of immune cells such as CTL. Naturally we could also try to use NK cells.

But findings from mouse studies suggest some bispecific antibodies can drive T cells into tumors, says Nai-Kong Cheung of Memorial Sloan Kettering Cancer Center. His lab has systematically tweaked design factors, such as how binding sites are arranged, to learn what optimizes the molecules' potency. And some companies hope to boost the attack on solid tumors with antibodies that bind not only to CD3, but also to another receptor on T cells known as a "second signal," which stimulates the cells to grow. For years, says Regeneron Senior Vice President Israel Lowy, industry has been "afraid to touch" that protein, called CD28, because of a

devastating mishap: An antibody designed to bind to it made six healthy volunteers critically ill from cytokine release syndrome in a 2006 U.K. clinical trial.

Findings from new studies, however, suggest it's possible to exploit that cell growth trigger safely.

Last year in Nature Cancer, a Sanofi team reported that a "trispecific" antibody with arms matched to CD28, CD3, and a cancer antigen wiped out myeloma tumors in mice[23].

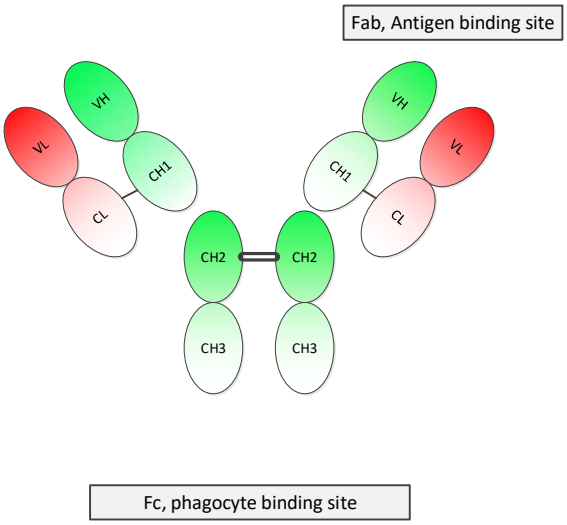
Other firms have split up the task by creating two bispecifics. One targets a tumor antigen and CD28 or another growth-signal receptor; the other binds to the tumor antigen plus CD3. "One of our hopes is that this costimulatory bispecific may help us unlock responses in solid tumors," says Lowy, whose company reported in Science Translational Medicine in January that such a two-drug combination shrank ovarian tumors and slowed prostate tumor growth in mice.

The above reference to tri-specifics is a critical observation. We shall return to this. Targeting CD28 and CD3 is but one of many targets. We shall also see that getting the correct targets will become the major challenge.

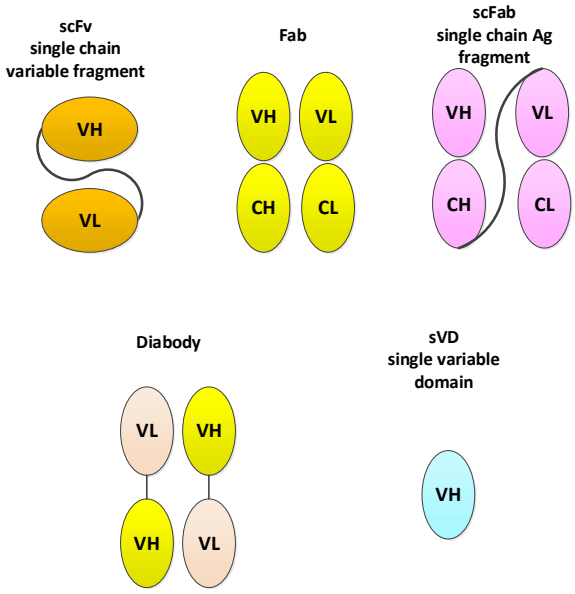
4.4 General Constructs

The Ab structures defined in this section can be called polyspecific antibodies. PAb. Let us start with a simple IgG antibody. It is shown below with Fab and Fc ends but also with a bond across the long chain in the middle. This Ab has a single Fc domain and thus attaching to a specific immune cells and a single Fab domain attaching to a specific Ab. The idea is that one can possibly create an Ab with multiple Ag attachments, and even ones where there is no Ac and immune attachment but all Ab attachments. Of course one could even imagine a set of poly Ag domains and thus we would potentially have a carrier that takes some molecule such as a therapeutic and then attaches to a specific cell such as a cancer cell.

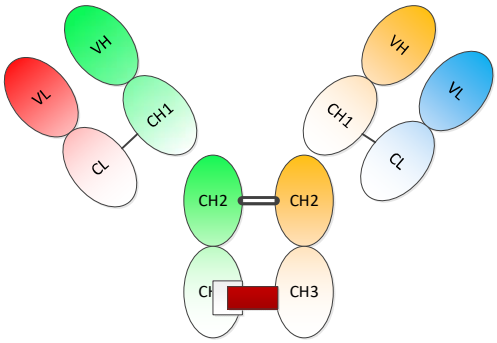
[23] See http://www.hcdm.org/index.php/molecule-information for lists of CD molecules.



The goal is to use the above paradigm but in the context of two different Abs from two differing hybridomas. We can have various ways or motifs to assemble them and the graphic below is an example.



Thus using these various motifs we can assemble a wide variety of bi-specifics. In fact these motifs can become the base set of any polyAb. Consider the modification of the classic IgG below:



Note: Knob is the red bump and hole is depression. They fit.
However 2 knobs or 2 holes will not fit

This is another variation called knobs in holes. Namely we have on the long end a solid binding protein extending outward while on the other side we have a protein inward and a matching of the proteins to lock in the structure. Furthermore in the above case we show a variety of long and short elements creating a complex motif. bi-specifics present a large multiplicity of shapes as well as binding locations.

4.5 Various Implementations[24]

We can now classify the variation is a variety of ways. The Table below look at IgG line, Fragment like and appended IgG or Fc. Frankly there may be many ways to classify b-specifics and we use a few different ones herein.

IgG Like Formats	Fragment Based	Appended IgG or Fc
κλ Bodies	VK/VL Format	Fv-IgG
Common LC	Single Domain	ScFv-IgG
Knob in Hole		Single Domain Ab-IgG
Charge Pair		BiTE
CH1/CL Cross Ab		DART

We now examine these in some shape detail.

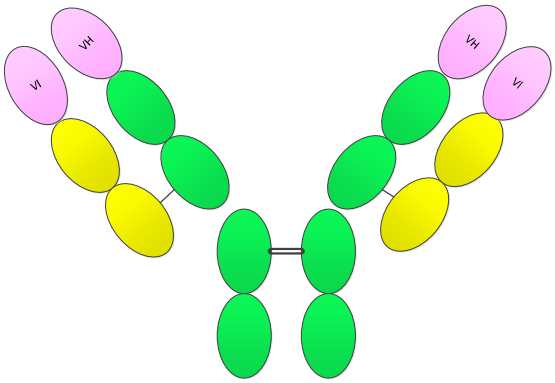
4.5.1 Fc Based Formats

The first division is Fc based which are the direct IgG like. Namely there is an Fc domain and Fab portions. We consider the various ones here.

4.5.1.1 Dual Variable Domains Ig (DVD-Ig)

[24] See Labrijn et al for a recent discussion and an extended interpretation

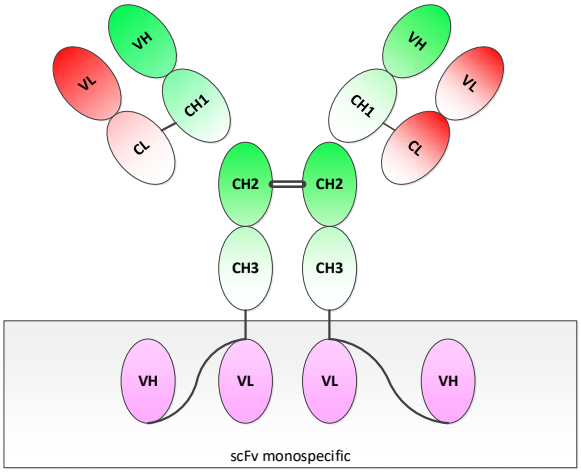
The DVD-Ig is shown below. This is a dual domain on both the Ab and Fc sides. The Ab sides have four variables due to the added binding domain. Note we have three on each Fab side rather than the two normally.



The above has been used in the case of binding VEGF and DLL4 ligands to inhibit angiogenesis in tumor cells[25].

4.5.1.2 scFv-Ig Fusions

This design is very complex in that it employs multiple motifs. It is symmetric but tetravalent.



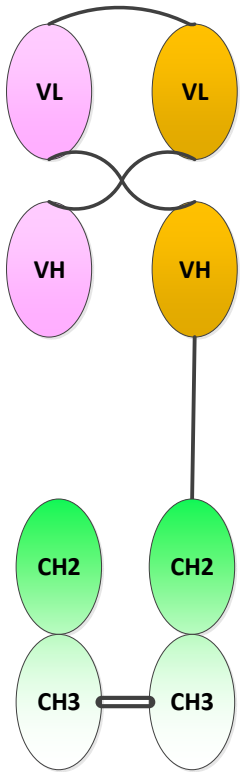
Currently this has been developed to target HER1 and cMET[26].

[25] Note: DLL4 is found to be a gene promoting hepatocellular cancer, see Kunanopparat et al, Delta-like ligand 4 in hepatocellular carcinoma intrinsically promotes tumour growth and suppresses hepatitis B virus replication, World J Gastroenterol 2018 September 14; 24: 3861-3870

[26] HER1 is also known as EGFR. The protein encoded by this gene is a transmembrane glycoprotein that is a member of the protein kinase superfamily. This protein is a receptor for members of the epidermal growth factor family. EGFR is a cell surface protein that binds to epidermal growth factor, thus inducing receptor dimerization and tyrosine autophosphorylation leading to cell proliferation. Mutations in this gene are associated with lung cancer. (see https://www.ncbi.nlm.nih.gov/gene/1956) cMET, also MET, encodes a member of the receptor tyrosine kinase family of proteins and the product of the proto-oncogene MET. The encoded preproprotein is proteolytically

4.5.1.3 scFv-Fc Fusions

scFv-Fc fusions is a fusion process extending the use of IgG structure Ab with more complex bonding. DART is an example. DART uses a fragment of Fcs as shown below, then has then fused with a diabody atop. The diabody is two chains interlinked and with DART they are further interlinked to yield stability. It is stated that this has the greatest stability due to this interlinking.

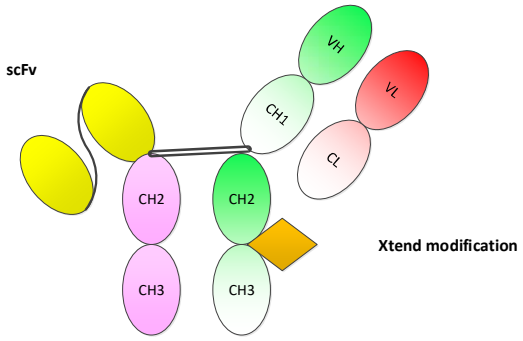


DART has the T cell targeting capacity due to the retaining of the Fc region and the variable ends allow for complex multi receptor binding. In effect this is a T cell guide Ab.

4.5.1.4 XmAb

processed to generate alpha and beta subunits that are linked via disulfide bonds to form the mature receptor. Further processing of the beta subunit results in the formation of the M10 peptide, which has been shown to reduce lung fibrosis. Binding of its ligand, hepatocyte growth factor, induces dimerization and activation of the receptor, which plays a role in cellular survival, embryogenesis, and cellular migration and invasion. Mutations in this gene are associated with papillary renal cell carcinoma, hepatocellular carcinoma, and various head and neck cancers. (see https://www.ncbi.nlm.nih.gov/gene/4233)

XmAb has an Fc domoain but there is an attached amino acid complex which alleges extends the lifetime of the Ab. The variable end is bi-specific with an scFv element and a standard format.



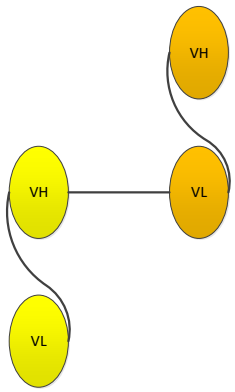
This has a Fab target of LAG-3 and a scFv target of CTLA-4[27].

4.5.2 Fragment Based, Fab

The second class is a non Fc based class of Fab variants.

4.5.2.1 BiTE

BiTE is a more mature bispecific. It contains the two motifs that we see below and no Fc element.



The Bispecific T cell approach has seen limited use. As Huehls et al note:

[27] LAG3 Lymphocyte-activation protein 3 belongs to Ig superfamily and contains 4 extracellular Ig-like domains. The LAG3 gene contains 8 exons. The sequence data, exon/intron organization, and chromosomal localization all indicate a close relationship of LAG3 to CD4. (see https://www.ncbi.nlm.nih.gov/gene/3902) CTLA-4 is a checkpoint protein and is targeted by many Abs in immunotherapy. his gene is a member of the immunoglobulin superfamily and encodes a protein which transmits an inhibitory signal to T cells. The protein contains a V domain, a transmembrane domain, and a cytoplasmic tail. Alternate transcriptional splice variants, encoding different isoforms, have been characterized. The membrane-bound isoform functions as a homodimer interconnected by a disulfide bond, while the soluble isoform functions as a monomer. (see https://www.ncbi.nlm.nih.gov/gene/1493)

Bispecific T cell engagers are a new class of immunotherapeutic molecules intended for the treatment of cancer. These molecules, termed BiTEs, enhance the patient's immune response to tumors by retargeting T cells to tumor cells. BiTEs are constructed of two single chain variable fragments (scFv) connected in tandem by a flexible linker. One scFv binds to a T cell-specific molecule, usually CD3, while the second scFv binds to a tumor-associated antigen. This structure and specificity allows a BiTE to physically link a T cell to a tumor cell, ultimately stimulating T cell activation, tumor killing and cytokine production.

BiTEs have been developed that target several tumor-associated antigens for a variety of both hematological and solid tumors. Several BiTEs are currently in clinical trials for their therapeutic efficacy and safety. This review examines the salient structural and functional features of BiTEs as well as the current state of their clinical and preclinical development….

The concept of using T cell retargeting for cancer therapy stretches back to the 1970s. Unlike macrophages, dendritic cells, and other accessory cells, T cells are present in copious numbers, expand rapidly upon activation, give robust and durable cytotoxic responses, and have the potential to generate immunologic memory. Furthermore, T cells have been found to attack tumors from the outside as well as infiltrating into the tumor. These features make T cells optimal therapeutic effectors for cancer. T cell redirection does suffer one significant challenge, which is the requirement of a second stimulatory signal to achieve full T cell activation and prevent anergy. Multiple bispecific formats have been developed to meet or circumvent this requirement.

Then Abbas et al also have noted:

Bispecific T cell engagers (BiTEs) facilitate the targeting of host T cells of any specificity to attack tumor cells. These reagents are recombinant antibodies engineered to express two different antigen binding sites, one specific for a tumor antigen and the second specific for a T cell surface molecule, usually CD3. In many of these antibodies, each antigen binding site is composed of a single chain variable fragment containing Ig heavy and light chain variable domains, similar to the CARs described earlier.

The presumed mechanism of action of BiTEs, based on in vitro studies, is the formation of immune synapses between the tumor cells and the T cells and the activation of the T cells by CD3 crosslinking. A CD19-specific BiTE is approved for treatment of acute lymphocytic leukemia. BiTEs specific for many other tumor antigens have been developed, including CD20, EpCAM, Her2/neu, EGFR, CEA, folate receptor, and CD33, and are at various stages of preclinical and clinical trials.

As Ross et al note:

For targets that are homogenously expressed, such as CD19 on cells of the B lymphocyte lineage, immunotherapies can be highly effective. Targeting CD19 with blinatumomab, a CD19/CD3 bispecific antibody construct (BiTE®), or with chimeric antigen receptor T cells

(CAR-T) has shown great promise for treating certain CD19-positive hematological malignancies.

In contrast, solid tumors with heterogeneous expression of the tumor-associated antigen (TAA) may present a challenge for targeted therapies. To prevent escape of TAA negative cancer cells, immunotherapies with a local bystander effect would be beneficial. As a model to investigate BiTE®-mediated bystander killing in the solid tumor setting, we used epidermal growth factor receptor (EGFR) as a target. We measured lysis of EGFR-negative populations in vitro and in vivo when co-cultured with EGFR-positive cells, human T cells and an EGFR/CD3 BiTE® antibody construct. Bystander EGFR-negative cells were efficiently lysed by BiTE®-activated T cells only when proximal to EGFR-positive cells.

Our mechanistic analysis suggests that cytokines released by BiTE®-activated T-cells induced upregulation of ICAM-1 and FAS on EGFR-negative bystander cells, contributing to T cell induced bystander cell lysis.

Namely the BITE approach is to create using an Ab a molecule which is CD3 on one end and say CD19 on the other and use this to cover a target and then to attract a T cell. In some ways this is akin to CAR-T where we place the receptor to the target on a T cell, here we use a T cell and attach the target to a known receptor on a T cell.

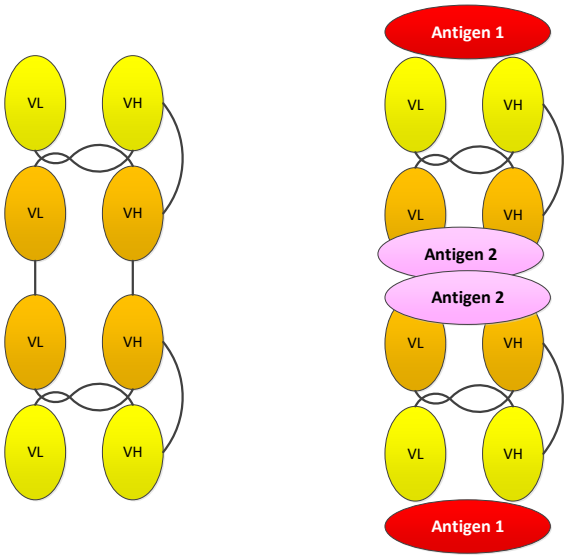
Furthermore, Zahavi and Weiner have recently noted:

Recently, the most successful mAb-based strategies have moved away from targeting tumor antigens and instead focused on targeting immune cells in order to enhance their anti-tumor capabilities. One of the first mAb approaches to stimulate T cell anti-tumor immunity was the development of bispecific T Cell Engager (BiTE) antibodies that both target a tumor antigen such as CD19 and the activating receptor, CD3, on T cells. BiTEs combine direct targeting of tumor cells with recruitment of cytotoxic T cells into the tumor microenvironment and led to tumor regressions even when administered at doses three orders of magnitude less than the parent mAb alone. The CD19-CD3 BiTE blinatumomab conferred significant clinical benefit to acute lymphoblastic leukemia patients and was FDA approved in 2017 .

Clinical trials are currently underway using BiTEs generated from the widely used anti-HER2 and anti-EGFR mAbs trastuzumab and cetuximab. Other mAb approaches seek to enhance T cell specific immunity against tumor cells by stimulating activating receptors such as 4-1BB, OX40, CD27, CD40, and ICOS. Agonist antibodies towards CD40 stimulate antigen presentation by dendritic cells and mAbs to OX40 and 4-1BB activate T cells while simultaneously dampening the activity of inhibitory T regulatory cells (Tregs) . mAbs designed to stimulate these activating receptors are in various stages of clinical trials both alone and in combination with other immunotherapy approaches. Additional mAbs that deplete inhibitory Tregs directly, such as daclizumab, which targets CD25 on Tregs, are also undergoing clinical trials

4.5.2.2 TandAb

TandAb is s homodimer consisting of four scFv motifs with linkers. Shown below it is a complex protein structure with multiple Ag binding sites.



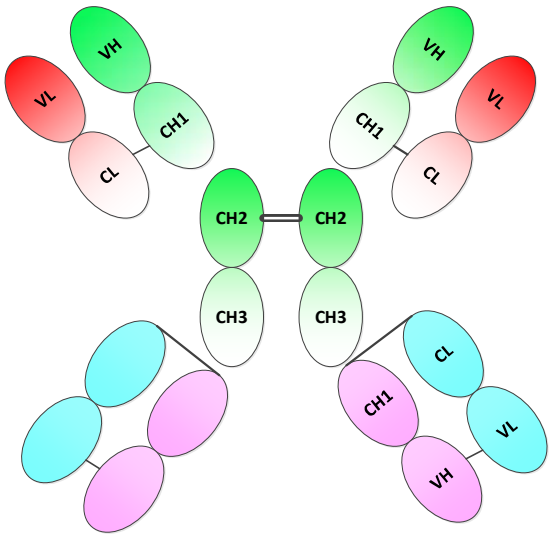
The TandAb form has been developed to block CD3 and CD19.

4.5.3 PreClinical

We now present a mix of preclinical polyAb.

4.5.3.1 biAbFabL

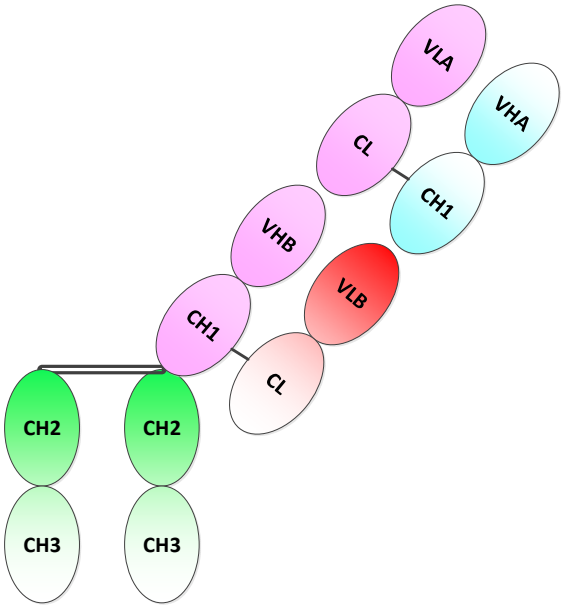
The biAbFabL is shown below and is composed of two Fab domains with a central C domain. Thus the Ab is tetravalent.



The targets for some current developments have been Killer cell Ig-like receptors (KIRs) Ig superfamily receptors expressed by NK cells that recognize different alleles of HLA-A, HLA-B, and HLA-C molecules. Some KIRs have signaling components with ITIMs in their cytoplasmic tails, and these deliver inhibitory signals to inactivate the NK cells. Some members of the KIR family have short cytoplasmic tails without ITIMs but associate with other ITAM-containing polypeptides and function as activating receptors. and IL-23 inhibition. These target a multiple set of inflammatory disease such as IBD, Chron's, MS and psoriasis.

4.5.3.2 MAT-Fab

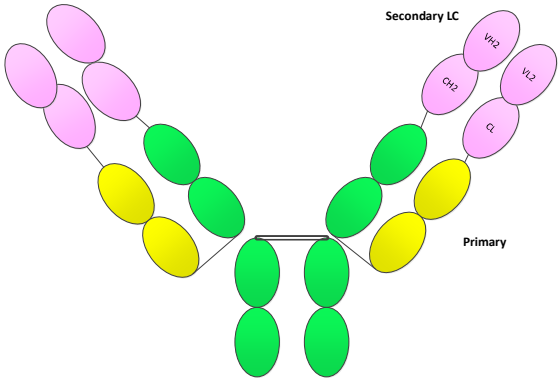
MAT-Fab is a complex tetrameric protein having four protein sections as shown below.



As with previous ones it targets T cells and also NK cells and macrophages. Some targets are CD3 on T cells as well as CD20 on specific cancer cells.

4.5.3.3 Tandem Forms

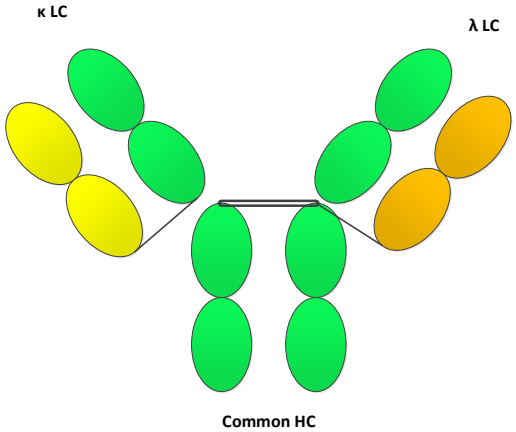
The Tandem form is a "Y-shaped" bispecific antibody format. It closely resembles that of standardized IgG antibodies, and, while being equipped with an Fc region and Fab regions, distinguished itself by having two sets of two Fab regions of different specificity linked in tandem in the Figure below. This enabled each form to retain moderately high to high binding affinity to both antigens. They are hence functional homodimeric tetravalent bispecific antibodies



The therapeutic design focuses on Toll Like Receptors, TLR, especially TLR 2 and TLR 4.

4.5.3.4 κλ antibody

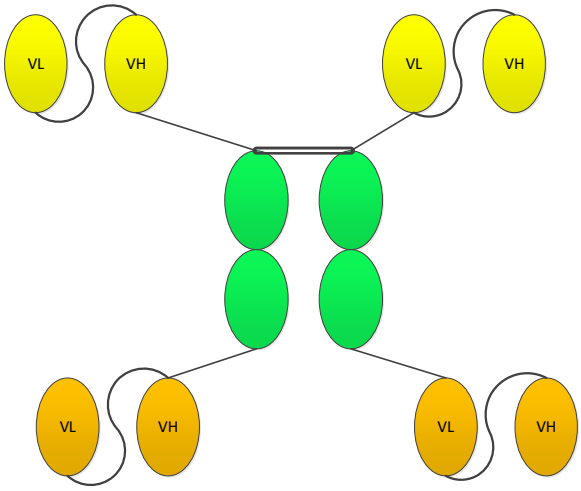
The antibody in this configuration is IgG like in structure except that it has two distinct Fab regions. These two light chains give bi-specific capability.



The therapeutic target is CD47 which appears on tumors and prevent T cell action. It blocks that target. It also blocks CD19

4.5.3.5 ADAPTIR

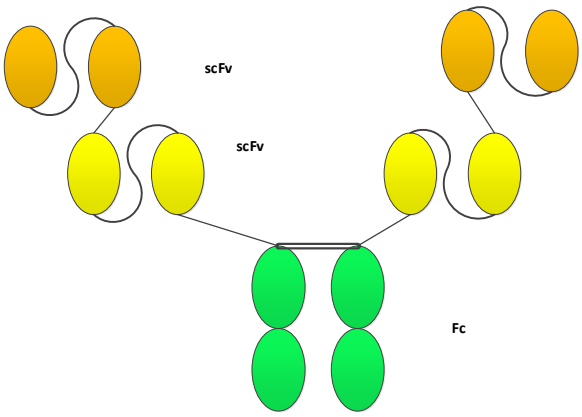
ADAPTIR as a bispecific antibody is comprised of an Fc region and four binding domains with two different specificities. The four binding domains are scFvs and attached in pairs at the amino and carboxyl ends of the Fc region. Thus, the Fc region has two binding domains at each end for binding two different antigens respectively, making it a tetravalent homodimeric bispecific antibody



The therapeutic target is a tumor necrosis factor 4-IBB and a tumor associated antigen 5T4. Targeting these two molecules with a bispecific antibody will promote potent tumor-directed immune T cell activation which makes ALG.APV-527 a potential drug for treatment of cancer.

4.5.3.6 BiIA-SG

This structure is a bispecific immunoadhesin bs-BnAb called BiIA-SG. It is an engineered immunoadhesin, which is an antibody-like molecule. It tetravalently binds to the two antigens via four scFvs fused to an IgG Fc region. It lacks the two CH1 domains that are native to the heavy chains of the IgG structure. The structure of the single gene-encoded BiIA-SG molecule is constructed using a gene tandem fusion method. This results in a structurally unique molecule with four scFv binding domains, two targeting HIV-1 gp120 receptor and 2 targeting human T cell CD4 receptor. The existing of two scFv for gp120 results in a significant higher binding affinity comparison to having only one.



This has been designed to treat HIV infections.

Jin et al present another set of these structures in their Figure 4.

4.6 Antibody Conjugates

Ab can target specific cells with specific surface Ag. We have demonstrated several of these options above. Now the Abs can also carry with them other elements such as chemotherapeutic molecules to be dropped into the targeted cancer cell. Jin et al have noted:

In recent years, the proposed use of ADCs has gradually gained steam, and they are rising stars in the tumor treatment field.

An ADC comprises three main components: a mAb, cytotoxic payload, and linker[28].

Upon binding with a target antigen on tumor cells, an ADC can deliver a cytotoxin payload into the targeted cell cytoplasm via receptor mediated endocytosis, release the cytotoxic drug from the ADC during lysosomal degradation to destroy DNA or otherwise inhibit cell division and eventually kill tumor cells. Suitable drug targeting, which is highly tumor-specific and readily internalized by cancer cells, is a key factor that determines the druggability of an ADC.31 To minimize on-target/off-target toxicity and open an acceptable therapeutic window for ADC applications, tumor-specific or overexpressed target antigens are preferable for ADC targeting and cytotoxic payload delivery.

Ideal ADCs are rapidly and efficiently internalized via the clathrin-mediated pathway and are efficaciously trafficked to lysosomes, where they rapidly accumulate.

[28] From Jin we have the definitions: *antibody-dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP).4 Inspired by the successful application of immunoglobulin G (IgG) mAbs, other antibody formats (e.g., antibody fragments, bispecific antibodies (BsAbs), and non-IgG scaffold proteins) and antibody derivatives (e.g., antibody–drug conjugates (ADCs) and immunocytokines.*

Currently, more than 50 antigens have been used as targets for the preclinical or clinical development of ADCs; these antigens include human epidermal growth factor receptor 2 (HER2), trophoblast cell-surface antigen-2 (Trop-2), and B cell maturation antigen (BCMA). The cytotoxic payload of an ADC is a highly potent drug capable of efficient cell killing. Compared with the effect of conventional chemotherapy, these payloads showed higher toxic potency (from 100- to 1000-fold). Free ADC payloads cannot be effectively administered as chemotherapy agent candidates due to their extreme potency, but their toxicities can be minimized by directing the potency of the cytotoxic payload by conjugating it to a tumor-specific antibody.

ADC payloads can be classified into two major types:

(i) tubulin inhibitors inhibit tubulin polymerization and trigger cell cycle arrest in the G2/M phase and subsequent cell apoptosis; these inhibitors include monomethyl auristatin E (MMAE), monomethyl auristatin F (MMAF), and a derivative of maytansine 1 (DM1).

(ii) DNA-damaging agents bind the minor groove in DNA, leading to cell death via DNA cleavage, DNA alkylation or interrupted DNA replication (these agents include calicheamicin, SN-38, DXd, and PBD).

Other small-molecule payloads, such as α- amanitin (a selective RNA polymerase II inhibitor), are also under investigation.

Linkers, which covalently conjugate the cytotoxic payloads to the antibody, are also essential components of ADCs.

Ideal linkers are stable before they reach the targeted tumor site and are rationally designed for rapid liberation of payloads from an ADC upon entry into lysosomes. Based on the mechanism of payload release, linkers can be categorized into cleavable or noncleavable linkers.

Cleavable linkers are designed to conditionally respond to the TME or intracellular environment, such as low pH (e.g., the acid-labile hydrazone-based linker in gemtuzumab ozogamicin (GO)), proteolysis (e.g., the valinecitrulline linker in brentuximab vedotin (BV)), or highglutathione concentrations (e.g., the disulfide linker in the maytansinoid-based ADC mirvetuximab soravtansine).

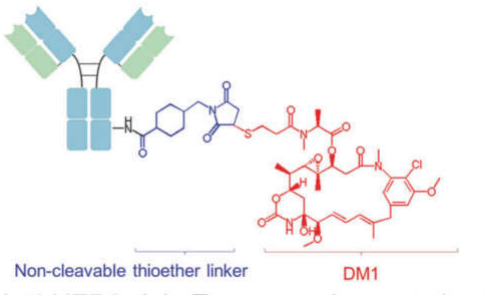
On the other hand, noncleavable linkers (e.g., the thioether linker in ado-trastuzumab emtansine) rely on complete lysosomal degradation of the antibody for payload release. The chemical conjugation strategies of ADCs play a significant role in the therapeutic potential of ADCs.

Usually, an ADC payload is conjugated to a surface lysine or cysteine residue of an antibody, resulting in the patterned distribution of ADCs with different drug-to-antibody ratios (DARs) during chromatographical separation*.*

Different DARs, which may vary from zero to eight, indicate different ADC pharmacokinetics, efficacy, and safety profiles.

Hence, site-specific conjugation approaches are being explored to generate homogeneous ADCs.

A useful ADC is one for HER2+ breast cancer, BCa. The structure is shown below. It is comprised of an antibody, transtuzumab, targeting the HER2 Ag on the tumor cell, and a ligand connected to a chemotherapeutic emtansine. We show this below from Jin et al:



Now Jin et al note:

Ado-trastuzumab emtansine. HER2 is overexpressed in approximately 20% of breast cancer patients.57 In 2013, Ado-trastuzumab emtansine (also known as T-DM1 or Kadcyla®, Genentech, Inc.), a ***HER2-targeting ADC incorporating the anti-HER2 trastuzumab*** *with the* ***microtubule inhibitor DM1 (a maytansine derivative) via a stable thioether linker****, was approved for the treatment of patients with HER2-positive metastatic breast cancer. The approval was based on a phase III trial (EMILIA). In the EMILIA trial, patients were randomly administered either T-DM1 (n = 495) or lapatinib plus capecitabine (n = 496). Median progression-free survival (PFS) (30.9 vs. 25.1 months; HR, 0.68; 95% CI, 0.55–0.85; p < 0.001) and median OS (9.6 vs. 6.4 months; HR, 0.65; 95% CI, 0.55–0.77; p < 0.001) were significantly better in the patients who received T-DM1 than in the patients who received lapatinib plus capecitabine. In addition to breast cancer, T-DM1 has been studied in patients with other solid tumors, including lung, bladder, brain, and colorectal cancer.*

5 NK TARGETING

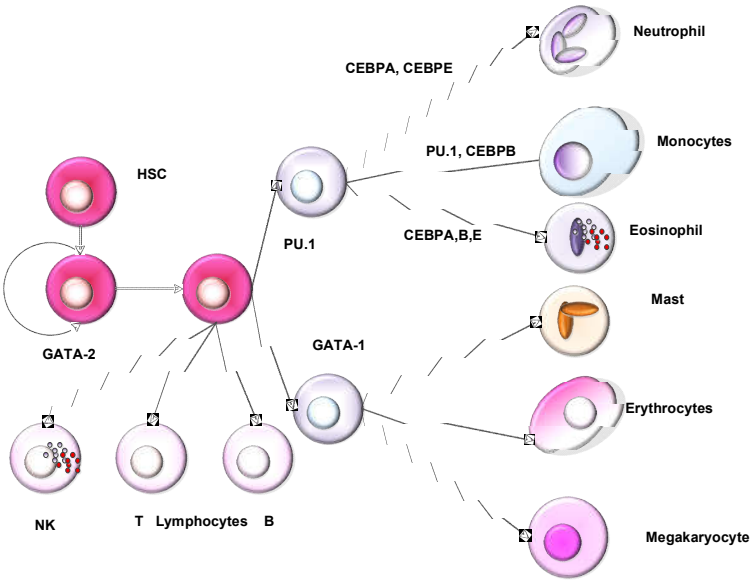
Prostate cancer has a complex immune environment. As Handa et al note :

Given the dynamic nature of immune cell types, the impact of the immune system on PCa is remarkably complex. Because high-grade PCa is characterized by low-level tumor infiltration of lymphocytes, the interactions between innate and adaptive immunity are not well understood. Macrophages and Treg cells have been associated with aggressive pathology, high rates of recurrence after prostatectomy, and worse distant metastatic free survival. On the other hand, mast cells, NK cells, and DCs are negatively associated with tumor progression, 1 and have been shown to confer improved distant metastatic-free survival.

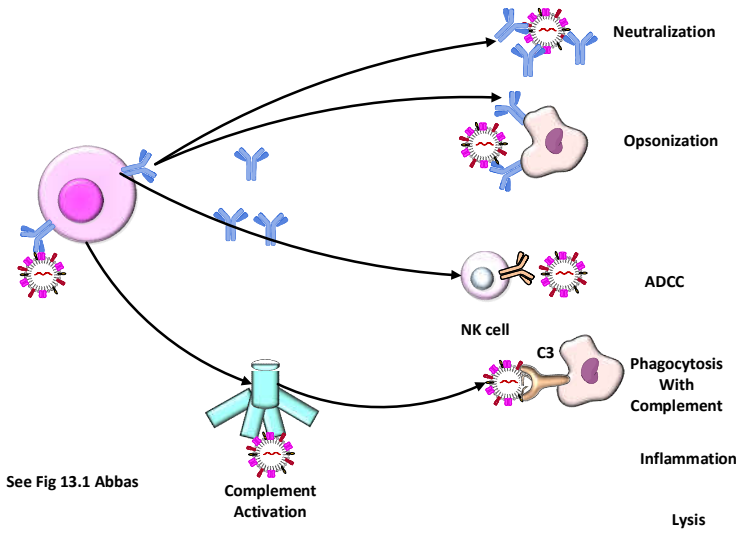
TILs in PCa may be dysfunctional and not capable of producing an immune response as suggested by examination of over 1500 resected PCa specimens wherein a greater TIL population was associated with a lower metastasis-free survival.77 Low tumor-associated antigen expression, DNA mismatch repair gene defects, subdued expression of MHC class I, lack of phosphatase and tensin homolog (PTEN) protein, and poor IFN1 signaling are some key processes that play a role in this complex tumor environment.

One study revealed that the average mutation frequency in PCa is almost 10 times lower than melanoma, which may explain the significant difference in response to immunotherapy between the two malignancies. Recent preclinical trials have shown that treatment with IFN-γ as well as radiation therapy, enhance MHC class I protein expression and may be associated with improved survival.123–125 The PTEN gene, which is known to be deleted in up to 30% of PCa cases, may be another emerging marker to assess immune responsiveness of PCa.126 For example, melanoma patients with PTEN loss have a much greater treatment response to PD-1 blockade.127 On the other hand, mutations in DNA damage repair (DDR) genes may in fact accord a survival benefit to patients treated with poly(ADP-ribose) polymerase (PARP) inhibitors such as Olaparib.

Thus when we examine the use of NK cells we must understand the interaction with the TME as well as all other immune cell interactions. The figure below depicts the keep immune cell players.



The types of attack on pathogens by immune cells can be graphically shown below:



5.1 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.

5.1.1 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[29]. 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 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.

5.1.2 NK

[29] See Abbas et al 4th Ed

Natural killer cells, NK, are elements of the innate immune system[30]. 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[31].

Based on the work of Lorenzo-Herrero et al:

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

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

Killer cell immunoglobulin-like receptors (KIRs)[32]

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

From Laskowski et al we have:

Despite the successes of engineered T cell immunotherapies1–8, the clinical benefit has been limited to a fraction of patients and a few indications, thus highlighting the need for new strategies. Leveraging innate immunity to broaden the scope of antitumour responses is an attractive option.

Within the innate immune system, NK cells are specialized immune effector cells, and are suspected to have a role in tumour immunosurveillance10, as suggested by the correlation of low

[30] See Islam et al for another summary

[31] See Abbas et al

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

NK cell activity with increased cancer susceptibility and higher risk of metastasis observed in both preclinical and clinical studies17–19. NK cells develop from CD34+ progenitor cells in the bone marrow, although it is as yet unclear whether they arise from a unique set of precursor cells or from multipotent progenitors that also give rise to T lymphocytes, B lymphocytes and myeloid cells20.

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

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

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

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

This broad range of attributes reveals the sophisticated network of biological mechanisms associated with NK cell function and supports the value of NK cells for immunotherapy. Memory-like function in NK cells. Early studies reported memory-like responses by NK cells in mouse models of cytomegalovirus infection, a behaviour not typically associated with innate immune cells. In these studies, mouse NK cells, when stimulated with a combination of IL-12 and IL-18, acquired a functional phenotype characterized by increased production of IFNγ.

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

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

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

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

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

As Raskov et al note :

Human NK cells are phenotypically characterized by the expression of neural cell adhesion molecule (NCAM/CD56) and the absence of CD3 and T cell receptors. They are mainly present in the lymph nodes and peripheral circulation where they constitute approximately 2 % of the leukocytes in the blood. The lifespan of human NK cells is approximately two weeks with an estimated doubling time in vivo of approximately two weeks. If stimulated continuously, peripheral blood NK cells can achieve up to 30 population doublings before entering senescence . In the defense against cancer, NK cells participate particularly in the immune surveillance of circulating tumor cells . Entering the circulation, the majority of tumor cells are eliminated by NK cells within 24 h; however, if and when the cancer advances, the activity of circulating NK cells generally decreases with disease progression .

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

The two major groups of NK receptors are the inhibitory killer-cell immunoglobulin-like receptors (KIRs) and the activating natural cytotoxicity receptors (NCRs) (see later). NK cells use dual mechanisms to kill their target: the direct killing process (natural cytotoxicity) and the antibody-dependent cell-mediated cytotoxicity (ADCC). In the former, the NK cell recognizes the activating ligands on the target cell surface (Fig. 1a). In ADCC, the NK cell receptor CD16

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

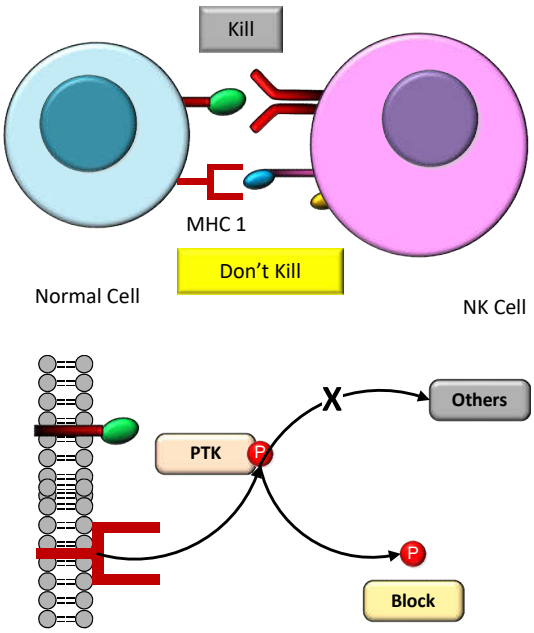
The following exocytosis of lytic granules results in immediate release of toxins (granzymes, granulysin, and perforin) into the immune synapse (Fig. 1b) and death receptor ligands, e.g. tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) engaging death-receptors on the target cell . The ADCC process cleaves the CD16 molecules from the NK cell surface and the recovery of CD16 takes several days. The shedding of the extracellular CD16 domain is caused by the proteolytic cleavage by disintegrins and metalloproteinases, and it may be an important factor in NK cell detachment from the target cell after the killing.

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

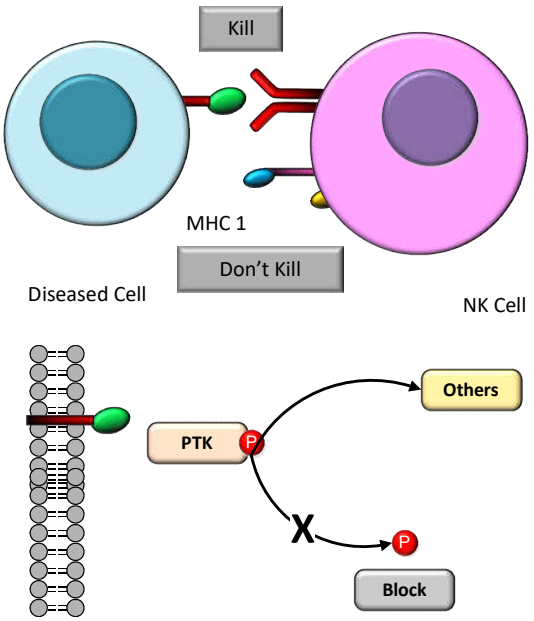
Ligand binding of CD16 on NK cells facilitates ADCC and lysis of target cells . For example, AFM13 is a novel NK cell-recruiting antibody that targets CD16A and CD30 and may provide a new treatment option for patients with relapsed or refractory Hodgkin lymphoma

The basic principles of NK activation is shown below:

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

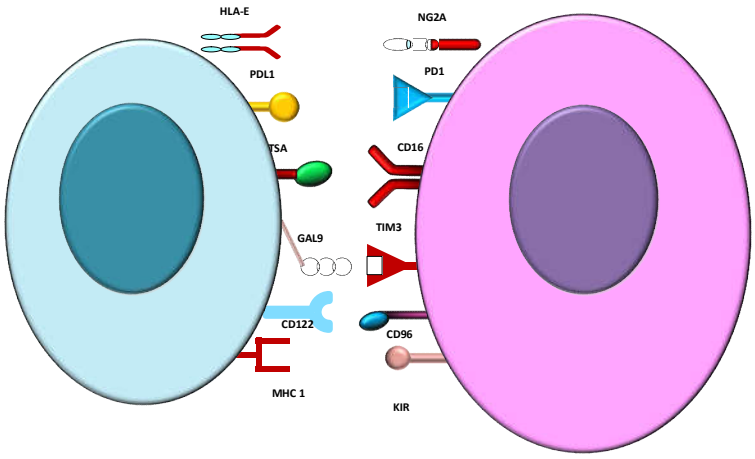


If however the Ag does not have an MHC 1 on the surface as below then we have a release and the cell is attacked. This we show below.



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

See Carotta



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

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

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

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

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

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

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

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

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

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

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

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

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

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

Finally, supported by the literature revision and our new findings on NK cell pro-angiogenic activities, we uphold NK cells to a key host cellular paradigm in controlling tumor progression

and angiogenesis; thus, we should bear in mind NK cells like a TME-associated target for anti-tumor therapeutic approaches.

As Lopez-Soto notes:

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

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

the secretion of IFNG; and

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.

5.1.3 NKT

The NKT 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

5.1.4 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 performin-ganzyme approach.

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

As Steer et al note:

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

tumour antigen(s) must be present, and

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

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

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

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.

5.1.5 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[33]. The CIK cells can be well targeted, well tolerated, and quite effective.

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

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33 https://www.researchgate.net/publication/280627292_MDS_METHYLATION_AND_THE_EPIGENETIC_PARADIGM

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.

The third general step is the use of CIK, or cytokine induced killer cells. These are somewhat akin to NK cells and have been developed specifically for cancers of these type. We briefly discuss how they are prepared. The efficacy is yet to be fully determined but there is a large base of Phase I and II Trials demonstrating efficacy.

Lin and Hui provide a definition for CIK cells:

Cytokine-induced killer (CIK) cells are polyclonal T effector cells generated when cultured under cytokine stimulation. CIK cells exhibit potent, non-MHC-restricted cytolytic activities against susceptible tumor cells of both autologous and allogeneic origins. Over the past 20 years, CIK cells have evolved from experimental observations into early clinical studies with encouraging preliminary efficacy towards susceptible autologous and allogeneic tumor cells in both therapeutic and adjuvant settings. … we anticipate that the continuous therapeutic application of CIK cells will likely be developed along two major directions: overcoming the challenge to organize large prospective randomized clinical trials to define the roles of CIK cells in cancer immunotherapy and expanding its spectrum of cytotoxicity towards resistant tumor cells through experimental manipulations.

Jiang et al add to this description as follows:

The number of immune cells, especially dendritic cells and cytotoxic tumor infiltrating lymphocytes (TIL), particularly Th1 cells, CD8 T cells, and NK cells is associated with increased survival of cancer patients. Such antitumor cellular immune responses can be greatly enhanced by adoptive transfer of activated type 1 lymphocytes.

Recently, adoptive cell therapy based on infusion of ex vivo expanded TILs has achieved substantial clinical success. Cytokine-induced killer (CIK) cells are a heterogeneous population of effector CD8 T cells with diverse TCR specificities, possessing non-MHC-restricted cytolytic activities against tumor cells. Preclinical studies of CIK cells in murine tumor models demonstrate significant antitumor effects against a number of hematopoietic and solid tumors. Clinical studies have confirmed benefit and safety of CIK cell-based therapy for patients with comparable malignancies.

Enhancing the potency and specificity of CIK therapy via immunological and genetic engineering approaches and identifying robust biomarkers of response will significantly improve this therapy.

Garafano et al have noted:

Cytokine induced killer cells (CIKs) are a heterogeneous population of polyclonal T lymphocytes obtained via ex vivo expansion of lymphocytes. They share phenotypic and functional characteristics with both, T cells and NK cells.

Initially described by Schmidt-Wolf et al. in 1991 , CIKs are efficiently expanded in vitro by incubation of peripheral blood mononuclear cell PBMCs with the timely addition of IFN-γ (1000 IU/mL) on day 0 of culture, mAb anti-CD3 (OKT3) (50 ng/mL) and IL-2 (500 IU/mL) on the next day, followed by the subsequent addition of IL-2 during culture . They possess a high proliferation rate and potent antitumor effects.

They are capable of exerting a potent MHC-unrestricted cytotoxicity against both hematologic and solid tumors, but not hematopoietic precursors and normal tissues.

Within the heterogeneous T cell population two main subpopulations can be distinguished, one coexpressing the CD3 and CD56 molecules (range: 40% to 80%), while the other presenting a CD3+ CD56- phenotype (range: 20% to 60%). It also comprises a small subpopulation (<10%) of CD3- CD56+ NK cells. The antitumor efficacy of CIKs has been reported to be associated with the CD3+ CD56+ subset . The addition of IFN-γ during generation of CIKs activates monocytes providing them with a contact-dependent factor CD58 (lymphocyte function associated antigen-3 LFA-3) and a soluble factor IL-12. These two factors are important for the expansion to CD56-positive T cells and the acquisition of T helper 1 phenotype of CIK cells . CIKs are able to secrete TNF-α, IL-2 and IL-6 but are not able to secrete IL-4, IL-7 and IL-12. Morphologically, CIKs are large and completely granulated.

They cannot be distinguished from NK cells. The complementary subsequent addition of anti-CD3 acts as a mitogenic stimulus and high doses of IL-2 principally promote the expression of the natural killer group 2 member D (NKG2D) transmembrane adapter protein DAP10, which in turn is essential for cytolysis .

Different strategies have been developed in order to improve the proliferation and efficacy of CIK cells by the addition of other soluble factors.

IL-15 plays an important role in the immune system. It is also able to further activate CIK cells .

A significantly increased anti-leukemic activity of CIK cells stimulated with the IL-15 modified protocol was observed compared to the conventional IL-2-activated CIK cells . IL-21 added to the cell culture increases anti-leukemic activity by enhancing the expression of perforin, granzyme B, FasL, IFN-γ and TNF-α . The use of CIKs clinically is widely facilitated by the reproducibility and simplicity of the expansion protocol and their significant MHC-independent antitumor efficacy against a broad range of cancers.

Compared to lymphokine-activated killer (LAK) cells which are induced by incubation with interleukin (IL) and tumor-infiltrating lymphocytes (TILs), CIK cells can be obtained more easily and reveal a higher tumor-specific cytotoxic activity. Although they are missing the Fcγ receptor CD16, which is a mediator of Antibody-dependent cell-mediated cytotoxicity ADCC mechanisms, it was observed in 2016 CIK cells to possess a donor dependent expression of CD16 which can have a strong effect both in vitro and in vivo .

However, a few groups did not observe any expression of this Fcγ receptor leading to some disagreement

The preparation and creation of CIK cells is done as described by Jakel et al:

*CIK cells are generated by culturing **peripheral blood lymphocytes (PBL)** with*

1. *interferon-γ **(INF-γ)** monoclonal*
2. ***antibody against CD3 (anti-CD3)** and*
3. ***IL-2** in a particular time schedule.*

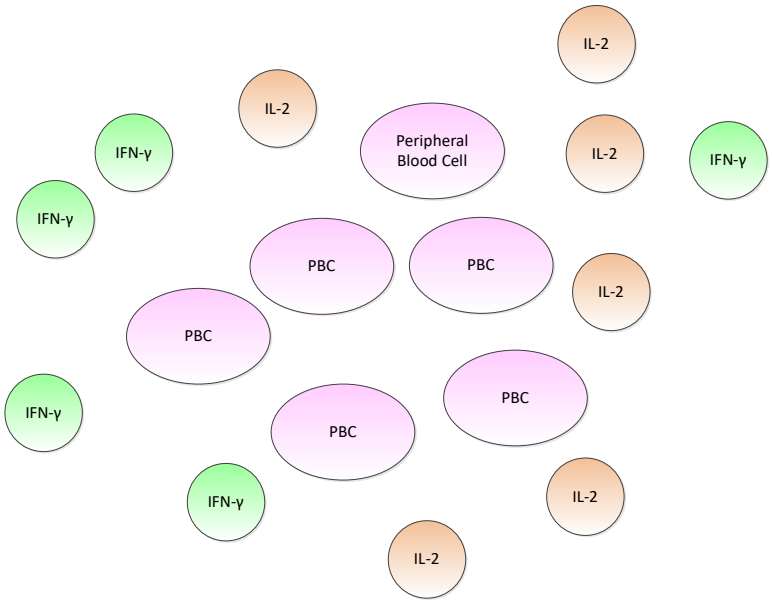
*The **cytokines INF-γ and IL-2 are crucial for the cytotoxicity** of the cells and anti-CD3 provides mitogenic signals to T cells for proliferation. Most of these CIK cells (87%) are positive for CD3 and for one of the T-cell coreceptor molecules CD4 (37.4%) or CD8 (64.2%), respectively.*

IFN-γ, added at day 0, activates monocytes providing crucial signals to T cells via interleukin-12 (IL-12) and CD58 (LFA-3) to expand CD56+ cells.

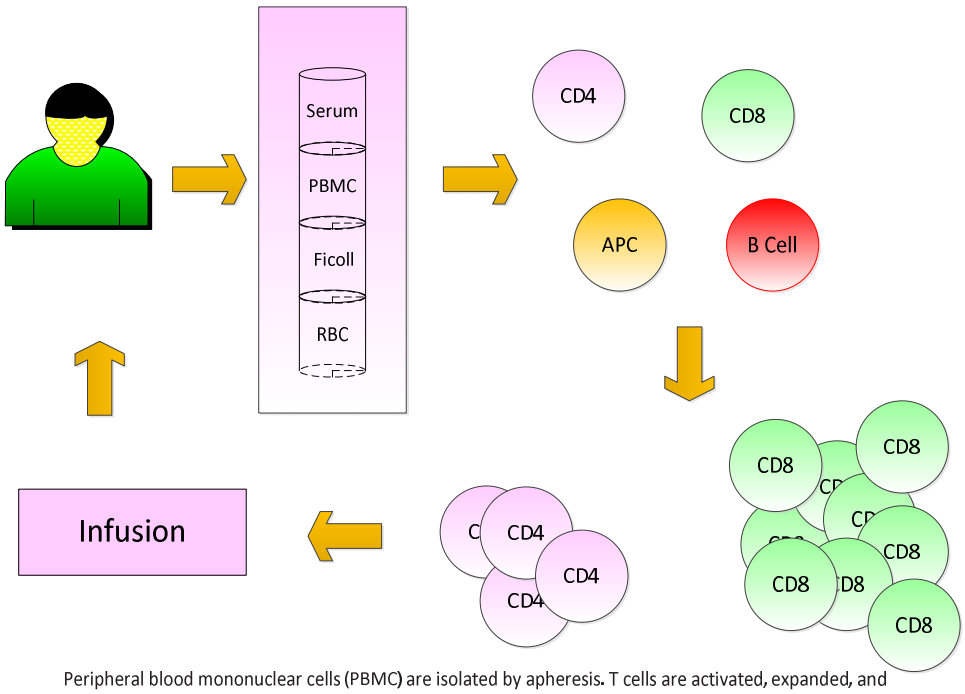
After 14 days of culture, 37.7% of cells are CD3+CD8+CD56+. These cells are referred to as natural killer T (NK-T) cells and represent the cell type with the greatest cytotoxicity in the CIK cell population.

Interestingly, these CD3+CD56+ double positive CD8+ T cells do not derive from the rare CD3+CD56+ cells in the starting culture but from proliferating CD3+CD8+CD56− T cells.

Their cytotoxicity is nonmajor histocompatibility complex (MHC)-restricted and they are able to lyse a variety of solid and hematologic tumors. Cell lysis is not mediated through FasL but through perforin release. CIK cell cytotoxicity depends on NKG2D recognition and signaling.



Jiang et al propose the following:



Peripheral blood mononuclear cells (PBMC) are isolated by apheresis. T cells are activated, expanded, and differentiated by anti-CD3 in the presence of cytokines including IFN-γ, IL-1α, and IL-2 for 14 to 21 days. These T cells, commonly called CIK, are then infused into patients. Jiang et al

Jiang et al prepare their cells as follows:

CIK cells have been evaluated as an adoptive cell immunotherapy for cancer patients in a number of clinical trials.

Peripheral blood mononuclear cells (PBMC) were isolated by apheresis.

T cells were then activated, expanded, and differentiated by

1. ***anti-CD3** in the presence of cytokines including*
2. ***IFN-γ,***
3. ***IL-1α, and***
4. ***IL-2***

*for **14 to 21** days to generate CIK, which were subsequently infused into patients.*

There are no significant clinical results for this in MDS but there are many Trials underway. One could suppose that this is a substantial third step after a BMT procedure. Logically it could be curative.

5.1.6 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.

5.1.7 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.

5.1.8 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.

5.2 NK Cells and Cancer

As Wlodarczyk and Pyrzynska have noted:

NK cells are large granular lymphocytes which are a component of the innate (nonspecific) immune defense system. They mature in the bone marrow, lymph nodes, spleen, thymus, and tonsils, and constitute about 5–10% of circulating lymphocytes in the peripheral blood (reviewed in). NK cells are essential components of the anti-tumor, antimicrobial, and anti-parasite defenses of our immune system. Most NK cells recognize antibody-coated tumor cells via their CD16 receptors (also known as FcγRIIIa), the first step that triggers the activation of antibody-dependent cellular cytotoxicity (ADCC).

Unlike T lymphocytes, the action of NK cells is less specific, which causes them to respond rapidly to the presence of transformed or infected cells without a prior need for antigen priming or induction of a specific immune response. However, the activation of NK cells is still strictly controlled by the integration of a variety of signals obtained by the activating and inhibitory receptors, as well as cytokine and chemokine receptors on the surface of NK cells (reviewed in).

For example, normal cells express MHC class I (MHC-I) molecules, and their proper amount on the cell surface inhibits NK cell activation via interaction with inhibitory killer immunoglobulin receptors (KIR) on their surface. In contrast, cancer cells synthesize a reduced amount of MHC I molecules, leading to reduced signaling from inhibitory receptors and, eventually, activation of NK cells.

6 CAR TARGETING

We now consider CARs. They have been available in one form or another for well over a decade[34].

6.1 CAR-T OPTIONS

CAR T cells are chimeric antigen receptors on T cells. Chimeric because one designs them specifically for the target cells and essentially crated a multiheaded receptor that matches the antigen presented by the tumor cell.

CAR-T cells are essentially engineered T cells, specifically cytotoxic T lymphocytes, CTL, engineered to target specific cells such as those in various hematopoietic cell lines. such as leukemias and lymphomas. There is no fundamental reason that they cannot be used for solid tumors but there are certain operational barriers which must be overcome.

As Kershaw et al note:

There are two main types of antigen receptors used in genetic redirection.

The first utilizes the native alpha and beta chains of a TCR specific for tumor antigen.

The second is termed a chimeric antigen receptor (CAR), which is composed of an extracellular domain derived from tumor-specific antibody, linked to an intracellular signaling domain. Genes encoding these receptors are inserted into patient's T cells using viral vectors to generate tumor reactive T cells….

The specificity of CARs is derived from tumor-specific antibodies, which are relatively simple to generate through immunization of mice. Recombinant techniques can be used to humanize antibodies, or mice expressing human immunoglobulin genes can be used to generate fully human antibodies. Single-chain variable fragments of antibodies are used in the extracellular domain of CARs, which are joined through hinge and transmembrane regions to intracellular signaling domains.

As Miller and Sadelain note:

The advent of gene transfer technologies, in particular those enabling the transduction of human T lymphocytes using gibbon ape leukemia virus envelope-pseudotyped g-retroviral vectors, created new opportunities for immune intervention based on T cell engineering. Patients' T cells, easily accessible in peripheral blood, can be genetically instructed to target tumors by transduction of receptors for antigen, utilizing either the physiological TCR or synthetic receptors now known as CARs.

[34] See https://www.researchgate.net/publication/325698155_Adoptive_Cell_Transfer (2018), https://www.researchgate.net/publication/309419224_CAR_T_Cells_and_Cancer (2016)

Both approaches have shown clinical successes, particularly in melanoma, targeting NYESO1, and in acute lymphoblastic leukemia, CARs are artificial, composite receptors for antigen that integrate principles of B cell and T cell antigen recognition. They are particularly attractive in that they elude human leucocyte antigen (HLA) restriction and are thus applicable to all patients irrespective of their HLA haplotypes, unlike TCRs. CARs may also overcome HLA downregulation by tumors, which deprives T cells of a ligand for their endogenous TCR.

The critical function of CARs is, however, not to merely target the T cells to a tumor antigen, but to enhance T cell function. Thus, effective CARs further integrate principles of T cell costimulation and provide a broad spectrum of functional enhancements acquired by directly soliciting selected costimulatory pathways

Juillerat et al note:

Adoptive immunotherapy using engineered T-cells has emerged as a powerful approach to treat cancer. The potential of this approach relies on the ability to redirect the specificity of T cells through genetic engineering and transfer of chimeric antigen receptors (CARs) or engineered TCRs1. Numerous clinical studies have demonstrated the potential of adoptive transfer of CAR T cells for cancer therapy but also raised the risks associated with the cytokine-release syndrome (CRS) and the "on-target off-tumor" effect.

To date, few strategies have been developed to pharmacologically control CAR engineered T-cells and may rely on suicide mechanisms. Such suicide strategies leading to a complete eradication of the engineered T-cells will result in the premature end of the treatment. Consequently, implementing non-lethal control of engineered CAR T-cells represents an important advancement to improve the CAR T-cell technology and its safety.

Small molecule based approaches that rely on dimerizing partner proteins have already been used to study, inter alia, the mechanism of T-cell receptor triggering15. Very recently, Lim and colleagues have adapted this approach to control engineered T-cells through the use of a multichain receptor.

Here, we describe a strategy to create a switchable engineered CAR T-cells. Our approach is based on engineering a system that is directly integrated in the hinge domain that separate the scFv from the cell membrane. In addition, we chose to implement this strategy in a novel CAR architecture that relies on the FceRI receptor scaffold.

The particularity of this design resides in the possibility to split or combine different key functions of a CAR such as activation and costimulation within different chains of a receptor complex, mimicking the complexity of the TCR native architecture. In this report, we showed that the hinge engineering approaches allowed to turn a T-cell endowed with an engineered CAR from an off-state to an on-state.

By controlling the scFv presentation at the cell surface upon addition of the small molecule, our system allowed to further induce the cytolytic properties of the engineered T-cell. Overall, this

non-lethal system offers the advantage of a "transient CAR T-cell" for safety while letting open the possibility of multiple specific cytotoxicity cycles using a small molecule drug.

Principles of T Cell Engineering and CAR Design

(A) Integration of B cell and T cell antigen recognition principles in the design of CARs. The heavy and light chain chains, which are components of the B cell receptor and Igs, are fused to the T-cell-activating z chain of the TCR-associated CD3 complex to generate non-MHC restricted, activating receptors capable of redirecting T cell antigen recognition and cytotoxicity.

(B and C) Integration of T cell activation and costimulation principles in dual signaling CARs designed to enhance T cell function and persistence in addition to retargeting T cell specificity. In

(B), the physiological abTCR associated with the CD3 signaling complex is flanked by the CD28 costimulatory receptor.

(C) shows a prototypic second-generation CAR, which comprises three canonical components: an scFv for antigen recognition, the cytoplasmic domain of the CD3z chain for T cell activation, and a costimulatory domain to enhance T cell function and persistence. Unlike the abTCR/CD3 complex, which comprises g, d, ε, and z signaling chains and is modulated by a multitude of costimulatory receptors, CARs possess in a single molecule the ability to trigger and modulate antigen-specific T cell functions.

6.2 GENERATIONAL ARCHITECTURE

There are currently three generations of CAR T cell design. We examine each here. As Cartellieri et al note:

In an attempt to extend the recognition specificity of T lymphocytes beyond their classical MHC-peptide complexes, a gene-therapeutic strategy has been developed that allows redirecting T cells to defined tumor cell surface antigens. This strategy uses both the cellular and humoral arm of the immune response by assembling an antigen-binding moiety, most commonly a single chain variable fragment (scFv) derived from a monoclonal antibody, together with an activating immune receptor.

Once this artificial immune receptor is expressed at the surface of a modified T lymphocyte, upon binding of the scFv to its antigen an activating signal is transmitted into the lymphocyte, which in turn triggers its effector functions against the target cell (Figure 2). In the first attempts to reconfigure T cells with antibody specificity the variable parts of the TCR α and β chains were replaced with scFv fragments derived from monoclonal antibodies. These hybrid T-cell receptors were functionally expressed and recognized the corresponding antigens in a non-MHC-restricted manner. As a consequence of the finding, that CD3ζ chain signaling on its own is sufficient for T-cell activation, the first "true" chimeric single-chain receptors were created by fusing a scFv directly to the CD3ζ chain. At that time this concept was called the "T body approach".

Nowadays these types of artificial lymphocyte signaling receptors are commonly referred to as chimeric immune receptors (CIRs) or chimeric antigen receptors (CARs).

The use of CARs to redirect T cells specifically against TAA-expressing tumor cells has a number of theoretical advantages over classical T-cell-based immunotherapies. In contrast to the long-lasting procedure of in vitro selection, characterization, and expansion of T-cell clones with native specificity for MHC tumor peptide complexes, genetic modification of polyclonal T-cell populations allows to generate TAA-specific T cells in one to two weeks. Engraftment with CARs enables T cells to MHC-independent antigen recognition; thus, major immune escape mechanisms of tumors such as downregulation of MHC molecules are efficiently bypassed.

Furthermore, proliferation and survival of modified T cells can be improved by the implementation of a multitude of signaling domains from different immune receptors in a single CAR

6.2.1 First Generation

Following Cartellieri et al we note regarding all three generations that:

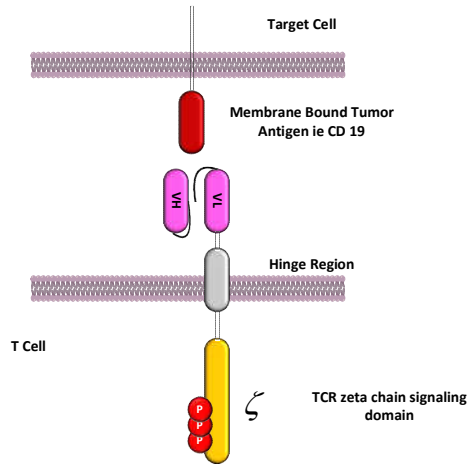
Evolution of CAR signaling capacities.

First generation CARs transmitted activating signals only via ITAM-bearing signaling chains like CD3ζ or FcεRIγ, licensing the engrafted T cells to eliminate tumor cells.

Second generation CARs contain an additional costimulatory domain (CM I), predominantly the CD28 domain. Signaling through these costimulatory domain leads to enhanced proliferation, cytokine secretion, and renders engrafted T cells resistant to immunosuppression and induction of AICD.

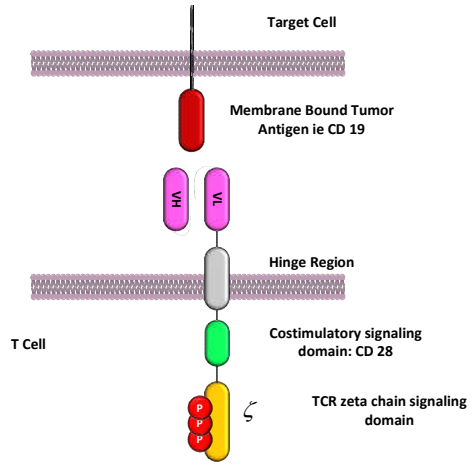
(Third Generation) Recent developments fused the intracellular part of a second costimulatory molecule (CM II) in addition to CD28 and ITAM-bearing signaling chains, thus generating tripartite signaling CARs. T cells engrafted with third generation CARs seem to have superior qualities regarding effector functions and in vivo persistence.

The first generation shown below is the simplest.



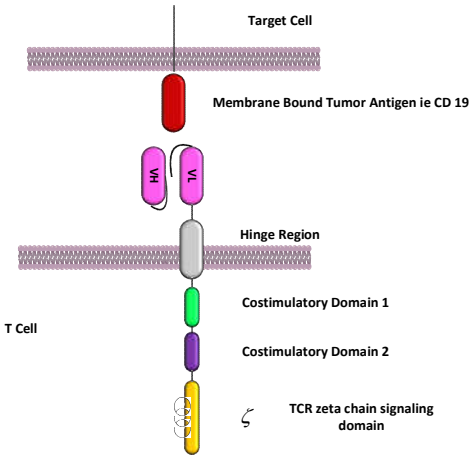
6.2.2 Second Generation

The second generation is as per below with the added element.



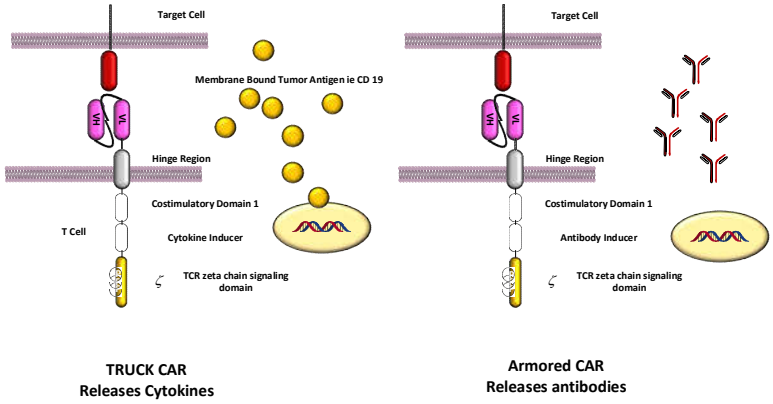
6.2.3 Third Generation

The third generation has added flexibility as shown below and described above.



6.2.4 Fourth Generation

The Fourth Generation CARS are a set of complex structures. There now are so many the two below are just a sample.



6.3 CAR-T EFFECTS

As Wlodarczyk and Pyrzynska have noted:

The recent innovative approaches to boost CAR-T cell function in TME are shortly summarized below …:

• TRUCK (T cells Redirected for antigen-Unrestricted Cytokine-initiated Killing) approach, based on engineering CAR-T cells to release particular transgenic cytokine upon CAR engagement, including IL-7 , IL-12 , IL-15 , IL-18 , IL-23 , and IL-33 . TRUCK CARs stimulate the release of cytokines specifically at the tumor site to provide either an auto-stimulatory effect for CAR-bearing cells or activation of other immune cell types in the TME;

• Armored CAR-T cells engineered to express various proteins alongside the CAR (reviewed in , such as antibodies or their fragments, which are able to inhibit immune checkpoints , or

dominant-negative TGF-β receptors , which are able to overcome TGF-β-induced T cell repression in the TME;

• Inducible CAR expression, regulated by specific cellular signaling and transcription factors, including synthetic Notch signaling , STAT5, AP-1, NFκB (to improve the control over timing and magnitude of CAR expression), or HIF-1α (to restrict the CAR expression to hypoxic areas of the solid tumors);

• ON- and OFF-controllable CAR signaling, regulated by clinically-approved drugs, including CAR regulated by lenalidomide-induced degradation , by dasatinibinduced downregulation and by proteolytical cleavage (to avoid CAR-T exhaustion and to obtain complete control over CAR activity by drug dosing);

• Multiplex CAR circuits combining various CAR technologies, including "AND gate," SUPRA CAR, universal ON-OFF, and switchboard VIPER CARs (for expanded control over CAR specificity and activation).

6.4 OTHER CAR OPTIONS

As Chen et al note :

CAR-T cell therapy has shown excellent clinical outcomes and has significantly transformed the treatment of various R/R hematological malignancies that previously have not had many treatment options. However, high treatment prices impose a substantial burden on patients and payers, thus hindering its commercial success. Furthermore, a high relapse rate, tumor antigen escape, and severe CAR-related toxicities are unresolved concerns. Nonetheless, the continuous development of CAR technology, novel CAR development and next-generation CARs, such as CAR-NKs and CAR-Ms, and CAR-based immunotherapy all have the potential to overcome the present restrictions and achieve a safer, more effective, and broader application in cancer treatment. Likewise, it is important that CAR-T therapy is affordable so that more patients can have access to it. This will help to increase our knowledge of the efficacy and safety of CAR-T therapy in practice.

6.4.1 CAR-NK

CAR-NK cells have the advantage of NK and CAR. The can be more readily produced and have greater generality. As El-Mayta et al note:

In recent years, immune cell-based cancer therapeutics have been utilized broadly in the clinic. Through advances in cellular engineering, chimeric antigen receptor (CAR) T-cell therapies have demonstrated substantial success in treating hematological tumors and have become the most prominent cell-based therapy with three commercialized products in the market.

However, T-cell-based immunotherapies have certain limitations, including a restriction to autologous cell sources to avoid severe side-effects caused by human leukocyte antigen (HLA)

mismatch. This necessity for personalized treatment inevitably results in tremendous manufacturing and time costs, reducing accessibility for many patients.

As an alternative strategy, natural killer (NK) cells have emerged as potential candidates for improved cell-based immunotherapies. NK cells are capable of killing cancer cells directly without requiring HLA matching.

Furthermore, NK cell-based therapies can use various allogeneic cell sources, allowing for the possibility of "off-the-shelf" immunotherapies with reduced side-effects and shortened manufacturing times.

Here we provide an overview of the use of NK cells in cancer immunotherapy, their current status in clinical trials, as well as the design and implementation of delivery technologies—including viral, non-viral, and nanoparticle-based approaches—for engineering NK cell-based immunotherapies.

As Wlodarczyk and Pyrzynska have noted:

The success of novel immunotherapeutic tools, such as CAR-T cells, has inspired scientists to focus on similar genetic modification of other types of cytotoxic lymphocytes, including NK cells. Additionally, there is a constant demand for exploring other lymphocytes as CAR platforms, since many patients treated with CAR-T still experience a progressive disease , severe therapy-related toxicities, or other adverse effects.

Most importantly, CAR-NK cells are considered an "off-the-shelf" product, as they can be produced from allogeneic sources, such as the blood of healthy donors.

Although NK cells have limited persistence in vivo and are more difficult to employ for genetic modification and expansion in vitro than T cells are, they are considered a safer, more powerful, and universal platform for CAR-based therapies due to their unique biological features … .

Importantly, CAR-NK therapies have the potential to be used as "off-the-shelf" types of therapy, since it has been documented that the HLA-mismatched NK cells can be employed as a CAR platform . As a matter of safety, CAR-NK therapy is characterized by less toxicity than CAR-T therapies, with no substantial GVHD or CRS effects . While administration of engineered T cells can cause the release of a variety of cytokines, among them IL-6, which is crucial for the occurrence of CRS (reviewed in), the NK cells produce mainly pro-inflammatory cytokines such as IFN-γ, IL-3, and TNF-α .

In the case of allogeneic NK cells' dose-limiting toxicities, CRS and GvHD are rarely observed, only after being administered in multiple doses or with other agents. For example, in the NSG mouse model, co-expression of CAR and IL-15 in NK cells, when administered in large amounts, resulted in toxicity, indicating the critical need to control NK cell survival in the body . In a recently published paper, expanded autologous NK cells were transduced with NKG2D-based CAR. Such cells exhibited in vitro cytotoxicity against multiple myeloma cells, with minimal

activity against normal cells. Mice injected with these CAR-NK cells did not show any sign of GvHD or treatment-related toxicities during the 150 days of the experiment .

Infusions of NK cells expressing IL-2, transduced with a lentiviral construct bearing a third-generation CAR which contained CD28 and 4-1BB co-stimulatory molecules, with an Fc fragment inserted between the CD33 scFv and CD28, have been safely used without significant side effects . Cord blood-derived anti-CD19 CAR-NK cells used to cure B-cell malignancies did not produce CRS or symptoms related to neurotoxicity.

As Chernosky and Tamagno have noted

A recent study showed that CAR-NK cells targeting PD-L1 expressed by Head and Neck Squamous Carcinoma (HNSCC) cells can successfully overcome the establishment of immune tolerance and eliminate tumor cells . The majority of clinical trials approved for CAR-NK cells are focused on hematologic malignancies rather than metastatic solid tumors, with only one trial currently evaluating the response of castration-resistant prostate cancer patients to CAR-NK cells targeting Prostate-Specific Membrane Antigen (PSMA) . Although CAR-NK cells show mild side effects and initial efficacy in lymphoid cancer patients , further studies are needed to evaluate short- and long-term effects of CAR-NK-based treatments in patients affected by advanced metastatic disease.

The NCI Trial database shows 9 trials for CAR-T and one for CAR-NK. The latter trial is in China.

6.4.2 Advantages of CAR-NK Cells

From Pfefferle and Huntington we have:

Adoptive cell therapy (ACT) with allogeneic haplo-identical NK cells has been clinically proven to be safe, without the risk of inducing GvHD. NK cells have been shown to be major contributors to the graft-versus-tumor (GvT) response observed after hematopoietic stem cell transplantation (HSCT) for acute myeloid leukemia (AML) .

Tumor escape via the loss of antigen or via the loss of major histocompatibility complex I (MHC-I) expression renders CAR-T cells helpless in detecting tumor cells . CAR-NK cells, on the other hand, retain their innate cytolytic capacity against germline-encoded tumor/stress ligands and are able to detect MHC-I-negative tumor cells due to their lack of a self-antigen . Decreased MHC-I expression is a characteristic ascribed to cancer stem cells (CSCs), which, together with the ligand expression of activating receptors, namely NKp30, NKp44, and NKG2D, is believed to sensitize CSCs to cytokine-activated NK cell-mediated killing [55–57].

Shedding of the NKG2D ligands MICA and MICB by breast CSCs and the absence of NKG2D ligands on leukemia stem cells are effective mechanisms utilized by CSCs to evade NK cell detection . Furthermore, NK cells have the ability to perform serial killing and although generally short-lived, cytomegalovarius (CMV)-induced memory-like adaptive NK cells have been shown to be long-lived and highly potent .

The safety profile of NK cells, combined with their anti-tumor potential, makes them a promising cell type for the implementation of CAR technology, which can redirect their cytotoxic potential towards a specific target. The feasibility of manufacturing an off-the-shelf CAR-NK cell product to universally treat patients would significantly increase the speed of administration, effectively reducing the lag time from the decision to treat and first dosing to 1 day.

As no severe toxicities are observed or expected with CAR-NK cells, treatment can be administered with 'out-patient' follow-up monitoring, significantly reducing the huge indirect costs associated with CAR-T cell therapy due to lengthy post-treatment hospitalization . While an induced pluripotent stem cell (iPSC)-derived CAR-T cell product can also be manufactured as an off-the-shelf product, extra genetic modification is required to remove the endogenous TCR in order to produce a universal product without the need for HLA-matching. Nonetheless, the serious side effects observed from CAR-T cell therapy would not be eliminated through the use of iPSC-derived CAR-T cells, retaining the need for post-treatment hospitalization.

6.4.3 CAR-M

Macrophages are also an immune vehicle to deliver an immunotherapeutic effect. As Wang et al have noted:

The chimeric antigen receptor (CAR) has become a promising approach to increase the cancer recognition capacity of immune cells. ***Based on their ability to penetrate solid tumours and traffic through the inhibitory TME, TAMs engineered with CAR constructs demonstrate sufficient potency.*** *Similar to CAR-T, the core components of CAR-M contain an extracellular domain that provides specific recognition by a single-chain variable fragment (scFv) (eg, CD19 and HER2), a hinge domain, a transmembrane domain (mostly CD8), and an intracellular domain that presents dedicated downstream signalling (eg, CD3z, FcgR).*

First-generation CAR-M cells are modified with edited CARs to target specific antigens to recognise tumour cells and improve their phagocytic ability. However, the CAR-M structure merely uses unique macrophage properties, mainly phagocytosis. As a representative structure, CAR-phagocytes (CAR-Ps) from Morrissey et al.1 were constructed to enhance phagocytosis.

The penetration capacity of macrophages was fully used in the original generation. Zhang et al.2 constructed CAR-HER2- CD147 to activate the expression of matrix metalloproteinases, which are stimulated by CD147 and can degrade the tumour extracellular matrix to overcome physical barriers.

Second-generation CAR-M cells are in development. In addition to maintaining the characteristics of first-generation CAR-M technology, the goals of second-generation therapies comprise improving tumour-associated antigen presentation and T cell activation.

The ideal method is to add an intracellular cytoplasmic domain to the CAR structure.

Second, the induction and maintenance of anticancer phenotypes to overcome the plasticity of TAMs must be considered.

Third, although macrophages cannot be expanded in vitro, we expected that the CAR-engineered macrophages would expand considerably and last a relatively long time to achieve satisfactory therapeutic effects in a small infusion dose. Based on these concepts, researchers have attempted to engineer murineorigin or human-origin bone marrow-derived macrophages to express CAR through chimeric vector transduction and then obtain the agents after expansion, concentration, and purification in vitro.

The anti-HER2 CAR-M (CT-0508) from Klichinsky et al. successfully demonstrated these improvements and has been evaluated in a first-in-human phase 1 clinical trial that focused on patients with recurrent or metastatic HER- 2 overexpressing solid tumours (NCT04660929).

Encouragingly, the United States Food and Drug Administration granted fast track designation to CT- 0508 in September 2021, which demonstrates the critical need for CAR-based TAM therapy development. However, the product cannot overcome the expansion obstacle, and the minimal effective dose requires further clinical investigation.

Likewise Pan et al have noted:

Adoptive cell therapy with chimeric antigen receptor (CAR) immunotherapy has made tremendous progress with five CAR T therapies approved by the US Food and Drug Administration for hematological malignancies.

However, CAR immunotherapy in solid tumors lags significantly behind.

Some of the major hurdles for CAR immunotherapy in solid tumors include CAR T cell manufacturing, lack of tumor-specific antigens, inefficient CAR T cell trafficking and infiltration into tumor sites, immunosuppressive tumor microenvironment (TME), therapy-associated toxicity, and antigen escape.

CAR Natural Killer (NK) cells have several advantages over CAR T cells as the NK cells can be manufactured from pre-existing cell lines or allogeneic NK cells with unmatched major histocompatibility complex (MHC); can kill cancer cells through both CAR-dependent and CAR-independent pathways; and have less toxicity, especially cytokine-release syndrome and neurotoxicity. At least one clinical trial showed the efficacy and tolerability of CAR NK cell therapy. Macrophages can efficiently infiltrate into tumors, are major immune regulators and abundantly present in TME.

The immunosuppressive M2 macrophages are at least as efficient as the proinflammatory M1 macrophages in phagocytosis of target cells; and M2 macrophages can be induced to differentiate to the M1 phenotype. Consequently, there is significant interest in developing CAR macrophages for cancer immunotherapy to overcome some major hurdles associated with CAR T/NK therapy, especially in solid tumors.

Nevertheless, both CAR NK and CAR macrophages have their own limitations. This comprehensive review article will discuss the current status and the major hurdles associated with CAR T and CAR NK therapy, followed by the structure and cutting-edge research of developing CAR macrophages as cancer-specific phagocytes, antigen presenters, immunostimulators, and TME modifiers …

CAR in CAR macrophages has the same structure as that in CAR T cells with an extracellular antigen-binding domain, hinge region, transmembrane domain and intracellular domain (Fig. 1). They differ in the intracellular signaling domain. CAR macrophages can directly use the CD3ζ intracellular domain as used in CAR T cells which contains immunoreceptor tyrosine-based activation motifs (ITAMs) [97–99]. In CAR T cells, ITAMs are phosphorylated by Src family kinases upon CAR engagement, bind to tandem SH2 (tSH2) domains in the kinase ZAP70, and activate CAR T cells to exert cytocidal effects. Macrophages do not express ZAP70.

They express another kinase Syk which contains tSH2 domains, can bind to CD3ζ and transduce phagocytic signals in macrophages . In addition to CD3ζ, other ITAM-containing intracellular domains, such as the γ subunit of Fc receptor (FcRγ) and multiple epidermal growth factor-like domains protein 10 (Megf10), have also been used and can induce comparable phagocytosis as CD3ζ . FcRγ transduces canonical signaling for antibody-dependent cellular phagocytosis (ADCP) in macrophages. Megf10 plays a critical role in phagocytosis of apoptotic cells by macrophages . Similar to the second- and third-generation CAR T cells, an additional signaling domain enhances phagocytosis. CAR T cells containing the CD3 domain without a co-stimulatory domain, as seen in the first generation CAR T cells, have limited in vivo activity .

Hence, all the FDA-approved CAR T products contain a costimulatory intracellular domain, either CD28 or 4–1 BB. Similar findings have been observed in CAR macrophages. It was previously reported that phosphoinositide 3-kinase (PI3K) signaling is important for phagocytosis of large particles . A tandem fusion of the CD19 PI3K-recruiting domain to CAR FcRγ tripled the phagocytosis of target whole cells .

Current status of CAR macrophage development-preclinical studies So far, CAR macrophage research is mainly at the preclinical stage with one Phase I trial ongoing which uses autologous CAR macrophages targeting HER2 overexpressing solid tumors. Morrissey et al. systemically analyzed intracellular domains that can be used to construct CD19- and CD22- targeting CAR macrophages .

CAR macrophages with any of the ITAM-containing intracellular domains, CD3ζ, FcRγ or Megf10, had comparable phagocytic efficiency while CAR macrophages containing Bai1 and MerTK intracellular domains could not bind to target beads.

Most CAR macrophages only internalized fragments of target cells, a phenomenon resembling trogocytosis or nibbling of live cells. Whole cell engulfment was infrequent. An antibody blocking the "don't eat me" signal CD47 or CAR containing FcRγ-PI3K-recruiting domains enhanced phagocytosis of whole cells, but still less than 10% of macrophages contained whole cells after 4–8 h of incubation. …

CAR macrophage is still at its nascent stage with only one clinical trial initiated and no results reported yet. Hence, many of the limitations have yet to be unfolded. Similar to CAR T and NK cells, CAR macrophages will need to go through 7 steps along the cancer-immunity cycle to achieve the cytotoxicity effects. Great endeavors are under way to optimize CAR macrophage structure, manufacturing, storage, tumor infiltration, and retention to cytotoxicity at TME. Repeated dosing may be needed to maintain sufficient CAR macrophage levels for active cancer surveillance.

One major advantage of using macrophages for ACT is its propensity in migration and infiltration into tumors . With the plasticity of inter-differentiation between pro-inflammatory M1 and anti-immune M2 phenotypes, high infiltration of macrophages into tumors and differentiation into the M2 phenotype can promote cancer growth and metastasis. Differentiation and retention of the M1 phenotype is being explored

7 OBSERVATIONS

We now consider several observation regarding extensions to the material covered above.

7.1 CELL SURFACE TARGETS, IDENTIFICATION AND ATTACK

Identify new targets and identifying existing targets are areas of significant interest. We have discussed several known targets but there is nothing to say that they are the only ones. This is especially true regarding stem cells. Also there may be a difference from patient to patients as well as over the course of the disease itself. We lack such information and it will be critical to assessing proper protocols.

Secondly we must have cost effective target measurements on a single cell basis. These should be part of a biopsy result. All too often we have genetic profiles of a mix of cells. Single cell profiling is critical and stem cell identification goes hand in-hand with single cell.

7.2 MORBIDITY ISSUES

Morbidity and/or adverse reactions are common amongst both Ab and immune cell therapeutics. In the case of Ab approaches, Hansel et al have detailed many results. They include:

1. Immune Response: *Acute reactions following infusion of mAbs can be caused by various mechanisms, including acute anaphylactic (IgE-mediated) and anaphylactoid reactions against the mAb, serum sickness, tumour lysis syndrome (TlS) and cytokine release syndrome (CRS). The clinical manifestation can range from local skin reactions at the injection site, pyrexia and an influenza-like syndrome, to acute anaphylaxis and systemic inflammatory response syndrome, which could be fatal.*

2. Infections*: Infectious diseases are a well-described side effect of certain mAbs, and they are a reflection of an acquired immunodeficiency, generally due to removal of the target ligand for that mAb. Indeed, particular types of infections illustrate the protective function of the target ligand in the normal immune system, and provide insights into the function of this molecule to combat particular pathogens*

3. Blood Disorders: *Drug-induced immune thrombocytopaenia can be caused by many medications, including mAbs93. An acute, severe, self-limiting thrombocytopaenia can be caused by infliximab (TNFα-specific), efalizumab (CD11aspecific) and rituximab (CD20-specific); however the mechanisms of action remain obscure.*

4. Autoimmune Diseases: *mAbs have the capacity through their immunomodulatory actions, including immunosuppression, to cause various autoimmune conditions*.

5. Dermatitis: *A well-known example for target-related rather than mAb-mediated adverse events relates to the human epidermal growth factor receptor 1*

6. Cancer: *Instead of excessive acute removal of malignant cells, some mAbs can contribute to tumour progression in a similar manner to other immunosuppressive agents. Association of TNF-specific mAb (infliximab) therapy with increased risk of malignancy remains controversial*

7. Cardiotoxicity: *Trastuzumab (Herceptin; Genentech) is a humanized mAb directed against human ERBB2 (also known as HER2/neu), and has been used successfully in women with ERBB2-positive metastatic breast cancer 151. However, an unexpected adverse event in women treated with trastuzumab in clinical trials was that of cardiotoxicity.*

8. Cytokine Storm: *various mAbs trigger the release of a range of cytokines, causing a cytokine storm or CRS*

As Chen et al note :

The extent of cytokine release reflects the degree of T-cell activation, which is crucial to achieving sufficient clinical effects. Nevertheless, it is also the cause of immune-related adverse events (irAEs), which includes CRS and neurotoxicity. Therefore, the strategy for preventing cytokine-related toxicity is to either diminish T-cell activity or block cytokine effects. Recently, a panel of experts that included oncologists, neurologists, cardiologists, and emergency medicine specialists reviewed the available studies for CAR-T and the irAEs developing as a result of the treatment with CAR-T.

The outcome of the discussion was published as the American Society of Clinical Oncology (ASCO) guidelines for the management of irAEs in patients treated with CAR-T therapy. The guidelines are intended to support the treating physician in managing the most common irAEs occurring during treatment with CAR-T, which includes CRS, immune effector cell-associated neurotoxicity syndrome (ICANS), infections, B-cell aplasia, and cytopenia. Management of CRS was mentioned above. Other serious irAEs that can occur are neurological toxicities, which are also known as ICANS.

They usually occur after four days of therapy . ICANS is the second serious irAE that is not infrequent in patients treated with CAR-T cells. The patients suffer from encephalopathy with other different symptoms, e.g., behavioral changes, aphasia, fine motor impairment, and headache . Severe cases might also require admission to the ICU in order to control seizures and intubating the patient if needed to avoid any damage to the airway passages. ICANS can occur with CRS or alone, and it can occur up to 1 month after the treatment with CAR-T. It can be self-limited, with symptoms resolving by 17 days, or may become severe and cause permanent neurological damage. Treatment of ICANS includes the administration of corticosteroids and supportive care.

Tocilizumab is contraindicated for the treatment of neurotoxicity, as it can worsen the symptoms. As CRS can occur with ICANS and CRS necessitates the treatment of tocilizumab, in accordance with ICANS, if it is low-grade and spontaneously resolves, the treatment of CRS can start and tocilizumab can be used. If the ICANS is severe and requires active measures, then managing ICANS is the first priority, before management of the CRS.

7.3 DORMANCY, A CANCER GRANULOMA?

Cancer cells appear to have an ability to become dormant and then after a period become active again. Thus the delayed metastatic effect.

As Chernosky and Tamagno have noted:

In pre-clinical models of breast cancer, tumor cells can seed within secondary organs at early stages of the disease, undergo dormancy, and re-awaken later to drive metastatic disease . In mice bearing advanced melanoma, metastatic tumor cells displayed early genetic divergence and contained unique mutations when compared to the tumor of origin, thus supporting the hypothesis of early dissemination and subsequent parallel transformation .

A study designed to track metastatic cells in a mouse model of pancreatic cancer showed that metastatic dissemination can occur unexpectedly early, even before the detection of a primary tumor . Dormancy, therefore, plays a fundamental role in metastatic relapse, as dormant cancer cells can survive for years before they are reactivated to drive recurrent, metastatic disease. For the most part, the pattern of reactivation of dormant tumor cells appears to be unpredictable, with tumors recurring as advanced metastatic disease decades after successful treatment. Up to 20% of breast and 45% of prostate cancer patients develop metastatic disease 10 years or more after successful eradication of the primary tumor .

It is now well documented that migratory tumor cells find an environment that supports a prolonged dormant state in several tissues, including Bone Marrow (BM) , microvasculature of metastatic sites , and lymph-nodes , depending on the cancer subtype. Evolving concepts of dormancy are providing clarity to two major challenges in oncology: asymptomatic minimal residual disease (MRD) and cancers of unknown primary (CUP) .

*MRD is asymptomatic and often undetectable; however, small clusters of * remain within secondary sites throughout the body and— upon specific stimuli, including increasing chronic inflammation and immune suppression—can emerge as recurrent, metastatic disease.*

They continue:

There are three major conditions that must occur to allow for tumor cell intravasation: angiogenesis, Epithelial-Mesenchymal Transition (EMT) of the tumor cells, and suppression of anti-tumorigenic immune cells. Tumor-secreted GM-CSF, CXCL12, and CCL2 attract monocytes to the TME and drive their differentiation into M2 TAMs, which then promote angiogenesis through secretion of Matrix Metalloproteinase 9 (MMP9) and Vascular Endothelial Growth Factor A (VEGF-A) [23–29]. Major Histocompatibility Complex (MHC) Class I expressed on the surface of tumor cells engages with Leukocyte Immunoglobulin Like Receptor B1 (LILRB1) on the surface of M2 TAMs to inhibit macrophage phagocytosis of the tumor cells

Finally they note:

Having established that the micro-metastatic niche—comprised of tumor cells, immune cells, and other tissue stromal cells—creates a new and unique microenvironment, we will next discuss how the evolution of the dormancy-associated microenvironment can lead to the reactivation of cancer cell expansion and macro- metastatic outgrowth. Several factors released within the TME exert opposing actions: either mediating or reverting dormancy. Depending on the subtype of solid tumor, dormancy can be prevented or reverted upon secretion of TGF-β1 and Periostin (POSTN) by sprouting endothelial cells. …

Neutrophils are among the cells of the innate immune system to mediate chronic inflammation in patients and have a specific role in facilitating the re-awakening of dormant cells. *Sustained experimental lung inflammation in mice, through either intra- nasal instillation of LPS or prolonged exposure to smoke, causes the reactivation of dormant breast cancer cells seeded in the lungs . remodeling triggered by neutrophils can activate Integrin α5β1-mediated signaling within dormant cells, thus promoting their proliferation. Integrin α5β1 has been previously associated with awakening dormant cells through its activation of Extra-Cellular Signal-Regulated Kinase (ERK) .*

ERK signaling is inhibited in dormant cells and is counterbalanced by strong activation of the p38 Mitogen-Activated Protein Kinase (MAPK); the ERK-p38 balance is an important determinant of dormancy. Upon Integrin α5β1 activation, p38 is inhibited, and ERK is activated, resulting in the resumption of proliferation . However, there is insufficient research dedicated to identifying what activates p38 to maintain a state of quiescence in dormant DTCs.

7.4 THE COST OF A CURE?

All of these personalized approached are labor intensive and thus costly. In many ways it is akin to CBCs before automated systems. Thus there will be a need for massive automation, achievable, but needs development.

As Chen et al note :

The notable outcomes of CAR-T cell therapy were considered a clinical success; however, on the commercial side, CAR-T therapy has achieved minimal success. The cost of CAR-T cell therapy is a significant barrier to patient access due to the complicated, highly personalized, and time-consuming manufacturing procedure. For example, for a single infusion, Kymriah costs $475,000, and Yescarta costs $373,000, excluding hospitalization for treatment side effects . Such a high price is a financial burden on both individuals and the healthcare system and limits the access of CAR-T to patients who need it.

If we can reduce the costs by an order of magnitude we then fit within the typical chemotherapeutic price range with better efficacy and dramatically reduced morbidity.

7.5 SOLID TUMORS AND THE CHALLENGE

Solid tumors have been a challenge for CAR. It would be the same for NK and other modalities. However we believe the targeting approach may reduce that threshold.

As Rafiq et al have noted:

In contrast to the striking successes achieved with CAR T cells in the treatment of patients with haematological malignancies, no equivalent successes have been demonstrated to date in patients with solid tumours, which collectively account for ~90% of cancer-related deaths.

The disappointing results in patients with solid tumours can be attributed to several factors; the lack of suitable tumour-specific antigens — or TAAs with expression profiles that are likely to be associated with tolerable on-target, off-tumour toxicities — is an obvious barrier to effective CAR T cell therapy for solid tumours. Nevertheless, the collective lack of efficacy observed with diverse CAR T cell products targeting several different solid tumour antigens suggests the existence of general barriers that could potentially be surmounted with additional CAR T cell engineering.

The complicated structure and cellular milieu of solid tumours influences both tumour biology and response to therapy. Solid tumours reside in tissues with lower numbers of endogenous T cells than lymphoid tissues and perhaps also lower levels of homeostatic cytokines and other T cell-supportive factors normally derived from the bone marrow and lymph node stroma.

The structure of the solid tumour stroma can pose a physical barrier to CAR T cell penetration. In addition, suppressive immune cells, such as regulatory T (Treg) cells and myeloid-derived suppressor cells, and immunosuppressive ligands, such as programmed cell death 1 ligand 1 (PD-L1), present in the TME might all quell intrinsic antitumour immune responses as well as CAR T cell responses. Overcoming antigen heterogeneity in solid tumours. Numerous strategies have been developed to overcome the antigen heterogeneity of solid tumours, some of which mirror the aforementioned strategies to overcome antigen escape in haematological malignancies.

For example, anti-EGFR BiTEs have been shown to increase the efficacy of anti-EGFRvIII CAR T cells in mouse models of glioblastoma and also of antifolate receptor-α CAR T cells in preclinical models of ovarian, colon or pancreatic cancer. Several technologies have been developed to create universal CARs for which adapter elements are used as ligands to enable the targeting of multiple antigens with a single CAR T cell population.

For example, avidin-linked CARs (named biotin-binding immune receptors) in combination with biotinylated antibodies can be used not only to control CAR T cell activity similar to a safety switch but also to target multiple antigens, either sequentially or simultaneously. Similar approaches involve the use of CARs with scFvs that recognize a fluorescein isothiocyanate fluorophore conjugated to TAA-binding molecules in order to target multiple antigens simultaneously.

Likewise, CARs that incorporate FcγRs as the antigen-binding domain enable the use of therapeutic TAA-binding antibodies to target multiple antigens with a single CAR molecule. In the SUPRA (split, universal and programmable) CAR system, leucine zipper motifs are used to

match CARs (zipCAR) with free scFvs (zipFv), again enabling simultaneous targeting of multiple antigens as well as the inclusion of multiple antigen logic gates and attenuation of CAR T cell activation (the CAR T cells are only active when zipFv are present). These and other technologies might provide a means to successfully target heterogeneous solid tumours in patients while minimizing off-tumour toxicities.

7.6 Oncolytic Viruses, Viral targeting, another attack mode?

We briefly mention the use of viral targeting. This is still in a nascent stage but the capabilities developed in the COVID process may help here. Knowing targets we can develop a virus that attacks cells with that target. Viral technology has advanced considerably so that we now have a wealth of understanding on how to generate those viruses.

The possible advantage of a viral attack is that it can reproduce for some period of time until the immune system responds. That will be the challenge.

As Evgin and Vile note:

Oncolytic viruses (OVs) and adoptive T cell therapy (ACT) each possess direct tumour cytolytic capabilities, and their combination potentially seems like a match made in heaven to complement the strengths and weakness of each modality. While providing strong innate immune stimulation that can mobilize adaptive responses, the magnitude of anti-tumour T cell priming induced by OVs is often modest. Chimeric antigen receptor (CAR) modified T cells bypass conventional T cell education through introduction of a synthetic receptor; however, realization of their full therapeutic properties can be stunted by the heavily immune-suppressive nature of the tumour microenvironment (TME).

Oncolytic viruses have thus been seen as a natural ally to overcome immunosuppressive mechanisms in the TME which limit CAR T cell infiltration and functionality.

Engineering has further endowed viruses with the ability to express transgenes in situ to relieve T cell tumour-intrinsic resistance mechanisms and decorate the tumour with antigen to overcome antigen heterogeneity or loss.

Despite this helpful remodeling of the tumour microenvironment, it has simultaneously become clear that not all virus induced effects are favourable for CAR T, begging the question whether viruses act as valets ushering CAR T into their active site, or vandals which cause chaos leading to both tumour and T cell death. Herein, we summarize recent studies combining these two therapeutic modalities and seek to place them within the broader context of viral T cell immunology which will help to overcome the current limitations of effective CAR T therapy to make the most of combinatorial strategies

7.7 Tumor Micro-environment

The TME is a major protective environment for a malignancy. We have discussed this at length before and it has many dimensions. Thus when developing new therapeutics we must be aware of the challenge to get through that protective shell.

As Dzobo et al have recently noted (2013):

Tumorigenesis is a complex and dynamic process involving cell-cell and cell-extracellular matrix (ECM) interactions that allow tumor cell growth, drug resistance and metastasis. This review provides an updated summary of the role played by the tumor microenvironment (TME) components and hypoxia in tumorigenesis, and highlight various ways through which tumor cells reprogram normal cells into phenotypes that are pro-tumorigenic, including cancer associated-fibroblasts, -macrophages and -endothelial cells.

Tumor cells secrete numerous factors leading to the transformation of a previously anti-tumorigenic environment into a pro-tumorigenic environment.

Once formed, solid tumors continue to interact with various stromal cells, including local and infiltrating fibroblasts, macrophages, mesenchymal stem cells, endothelial cells, pericytes, and secreted factors and the ECM within the tumor microenvironment (TME). The TME is key to tumorigenesis, drug response and treatment outcome.

Importantly, stromal cells and secreted factors can initially be anti-tumorigenic, but over time promote tumorigenesis and induce therapy resistance. To counter hypoxia, increased angiogenesis leads to the formation of new vascular networks in order to actively promote and sustain tumor growth via the supply of oxygen and nutrients, whilst removing metabolic waste.

Angiogenic vascular network formation aid in tumor cell metastatic dissemination. Successful tumor treatment and novel drug development require the identification and therapeutic targeting of pro-tumorigenic components of the TME including cancer-associated- fibroblasts (CAFs) and -macrophages (CAMs), hypoxia, blocking ECM-receptor interactions, in addition to the targeting of tumor cells. The reprogramming of stromal cells and the immune response to be anti-tumorigenic is key to therapeutic success. Lastly, this review highlights potential TME- and hypoxia-centered therapies under investigation

7.8 INFLAMMATION

Inflammation has been known to be a driver for malignancies. Reducing inflammatory states may reduce risks but in the case of PCa we all too often see it as the most significant factor.

As Lee et al note :

Prostate cancer is one of the most challenging cancer types among men. Cancer progression and therapeutic resistance often lead to high mortality rates . Prostate cancer ranks as the second leading cause of cancer-related deaths among American men . Well-known risk factors for prostate cancer include older age , black race , BRCA mutations , and family history . Interest in the linkages between inflammation and prostate cancer has increased.

Chronic inflammatory disease, such as prostatitis, was proved to increase the risk of prostate cancer .

On the other hand, some negative associations between chronic inflammation and prostate cancer have also been reported . Knowledge of the role of inflammatory cytokines in prostate cancer progression may provide an optimized targeted-therapy strategy.

IL-30 overexpression by prostate cancer stem-like cells (PCSLC) promoted tumor onset and progression in vivo.

IL-30 also played a critical role in PCSLC spread to lymph nodes and bone marrow by increasing the CXCR5/CXCL13 axis and lung metastasis through the CXCR4/CXCL12 axis. In this study, suppressing PCSLC by the proper targeting of upstream drivers was suggested as a potential treatment against prostate cancer progression and recurrence .

Interestingly, TGF-β1 shows double-faced functions in prostate cancer progression. At early stages, it acts as a cancer growth inhibitor, while at advanced stages, it promotes cancer development .

Park et al. reported that TGF-β1 activates IL-6 in human prostate cancer cells via synergistic signaling pathways, which are Smad2, p38-NF- κB, JNK, and Ras. IL-6 accelerates cancer cell proliferation and survival, which influence the progression and metastasis of prostate cancer.

In addition, elevated IL-6 may contribute to the conversion of TGF-β10s role as a prostate cancer promoter. Anti-IL-6 neutralizing antibody or antisense IL-6 effectively inactivated IL-6 signaling, leading to TGF-β-mediated apoptosis .

Various clinical trials also showed the association between inflammatory cytokines and prostate cancer prognosis. First, IL-6 levels in serum are increased in patients with prostate cancer, and it significantly correlated with cancer prognosis. A clinical study from Nakashima's team measured IL-6 levels in serum samples from stages B, C, and D prostate cancer patients. In this study, high serum IL-6 levels were associated with the advanced stages of prostate cancer and poor survival rate .

IL-6 has been reported to increase erythrocyte sedimentation rate , which was proved to be a prognostic factor in the survival of advanced prostate cancer patients . Another study from Michalaki et al. reported elevated IL-6 and TNF-α serum levels in prostate cancer patients compared with healthy controls. These increased inflammatory cytokines were correlated with advanced stages, metastasis, and poor overall survival in patients with prostate cancer . TNF-α was also suggested to play an important role in the development of cachexia from prostate cancer patients.

The patients with high-TNF-α serum levels showed higher performance status and mortality rates than the patients with undetectable TNF-α serum levels . IL-17 is another inflammatory

cytokine overexpressed in prostate cancer. Steiner et al. performed a screening of inflammatory cytokines from normal, benign hyperplastic, and malignant prostate tissues.

IL-17 was rarely expressed in the normal prostate, whereas its expression was increased in benign hyperplastic and malignant prostates. In addition, a significant correlation was monitored between IL-17 level and both IL-6 and IL-8 levels in malignant prostate specimens.

7.9 SINGLE CELL GENOMICS

As we noted above, developing single cell genomic capabilities as well as reporting in an understandable manner is a sine qua non.

As Jofe et al have noted:

In our vision of the future of immunotherapy drug-development pipelines, single-cell genomic analysis will be fully integrated into multiple steps.

The initial step would be to assess the potential targets (cells, pathways and molecules) by analyzing the single-cell profiles of large collections of tumor samples of different oncological pathologies*.*

This analysis should first include the cell-typespecific expression of the drug target at the level of RNA and protein, with the identification of potential target cells by scRNA-seq coupled to protein quantification by FACS indexing or CITE-seq, in which the addition of antibody–oligonucleotide conjugates informs the protein-expression level at the single-cell level14. In addition, parallel single-cell technologies can be used for validation of these signatures, such as mass cytometry (CyTOF), which can profile the expression of dozens of protein per cell.

Next, correlations and anticorrelations of cell types and molecular signatures of the TME of patients who express the target would be carried out to better define the target's mode of action—for example, correlation to specific immune or stromal cells, specific gene signatures, the signaling milieu, spatial location and proximity to other immune cells, and receptor–ligand analysis to identify potential cell–cell interacting partners and signaling*.*

Once a potential target is identified, a pre-clinical model must be generated. Here too, single-cell genomics could have a dominant role, as it enables accurate comparison of the human TME and animal model TME by projection of single-cell data from the preclinical model with the cellular composition and activity of the TME in the human cancer on which it is modeled13.

For identification of the preferred pre-clinical model for testing of the agent, single-cell profiling of any potentially relevant pre-clinical models would be generated and compared with the human cell and molecular profiles, and the models with the best match would be selected. These would then be used for initial testing of the efficacy of newly developed agents and for head-to-head comparison of several optional agents and their combinations.

Both effective treatments and treatments to which the preclinical models are unresponsive would then be further investigated by single-cell profiling of tumors following treatment, to highlight the mechanism of action of the agents in question. Longitudinal time-course single-cell profiling of tumors from treated pre-clinical models would assist in the characterization of the direct target cells and pathways, as well as secondary effects manifested by the agents through the response of the on-target cell populations; this would thereby enable effective evaluation of various treatment courses and combinations.

Developing synergistic therapies by single-cell analysis

While immunotherapy has had a considerable effect on a large number of cancer patients, most patients do not respond to monotherapy of current first-line treatments, such as ipilimumab (monoclonal antibody (mAb) to CTLA-4) or pembrolizumab (mAb to PD-1). A growing concept among researchers in the immunotherapy field, which has practically become a consensus, is the huge potential of immunotherapy combinations.

Marking the way is the combination of antibody to CTLA-4 (anti-CTLA-4) with either anti-PD-1 or anti-PD-L1; such combinations have proven as effective single-agent treatments in cancers such as melanoma15 and other conditions and are now being tested in hundreds of trials (https://www.fda.gov). We argue that singlecell analysis must be used for better identification, in a data-driven approach, of effective combination therapies.

The molecular resolution of single-cell profiling can be used to delineate potential synergistic, additive, neutral or antagonistic cellular and molecular activities of combination immunotherapy in pre-clinical or clinical trials. For example, one study used scRNA-seq and CyTOF analysis to assess the treatment of mouse models with single therapy of anti-CTLA-4 or anti-PD-1 versus combination therapy with both.

Combination therapy increased the expansion of specific CD4+T cell and monocyte subsets, and also decreased the levels of specific macrophage populations relative to their abundance after single therapy alone. This study suggests that combination therapy may have alternative mechanisms of action resulting from crosstalk among cell populations that are affected by the single agents. More importantly, expanding this approach, comprehensive single-cell data of the TME before and after treatment can drive the development of data-driven, synergic treatments. For example, this strategy can be used for intelligent combination of anti-PD-1 or anti-PD-L1 with synergistic targets on myeloid or stromal types, which increases T cell tumor recognition, activation and infiltration, while inflammatory innate cells are recruited and suppressive myeloid cells are removed.

Combining anti-PD-1 with activation of the co-stimulatory receptor CD40 or other means of activating dendritic cells will prime T cells and promote their tumor recognition and infiltration of the TME and will thereby increase the activity of anti-PD-1 in patients with small amounts of tumor-specific T cells at baseline.

Combination therapy that includes an antagonist of the TME inhibitory cytokine milieu produced by stromal cells, such as TGF-β, can potentially increase anti-tumor activity in specific tumors

with low infiltration by and/or activation of T cells. Strategies that combine checkpoint blockade with depletion of myeloid-derived suppressor cells may enhance the activity of anti-PD-1 and/or anti-PD-L1 in patients who do not respond to monotherapy alone

7.10 VACCINES

Vaccines have become of more interest as we better understand how they function as well as how to mass produce them.

As Badrinath et al note:

Most cancer vaccines target peptide antigens, necessitating personalization owing to the vast inter-individual diversity in major histocompatibility complex (MHC) molecules that present peptides to T cells.

Furthermore, tumours frequently escape T cell-mediated immunity through mechanisms that interfere with peptide presentation1. Here we report a cancer vaccine that induces a coordinated attack by diverse T cell and natural killer (NK) cell populations. The vaccine targets the MICA and MICB (MICA/B) stress proteins expressed by many human cancers as a result of DNA damage. MICA/B serve as ligands for the activating NKG2D receptor on T cells and NK cells, but tumours evade immune recognition by proteolytic MICA/B cleavage. Vaccine-induced antibodies increase the density of MICA/B proteins on the surface of tumour cells by inhibiting proteolytic shedding, enhance presentation of tumour antigens by dendritic cells to T cells and augment the cytotoxic function of NK cells.

Notably, this vaccine maintains efficacy against MHC class I-deficient tumours resistant to cytotoxic T cells through the coordinated action of NK cells and CD4+ T cells.

The vaccine is also efficacious in a clinically important setting: immunization following surgical removal of primary, highly metastatic tumours inhibits the later outgrowth of metastases. This vaccine design enables protective immunity even against tumours with common escape mutations

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