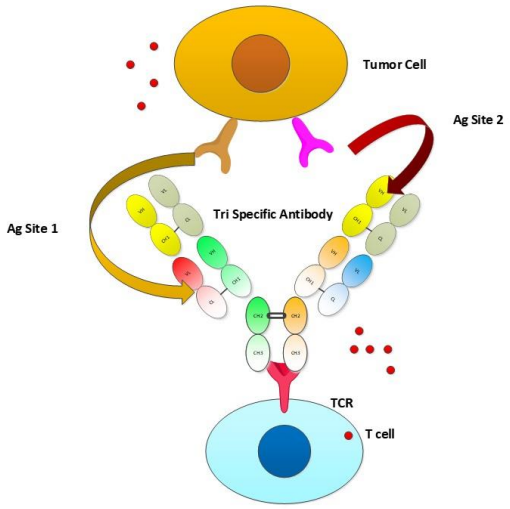

POLY SPECIFIC ANTIBODIES

ABSTRACT

TERRENCE MCGARTY

This Report details the current efforts on the development of poly-specific antibodies (PolyAb) which are logical extensions of monoclonal Ab. PolyAb present an interesting tool to address a variety of disease directly and via the transmission of therapeutics.

TGL 181, DECEMBER 2020

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Contents

1 INTRODUCTION

Antibodies have been known for decades. The approach of using IgG infusions as a means of dealing with infections and other diseases was a classic shotgun approach. Namely antibodies (Ab) work, at the time no one really knew why of how, but one could isolate IgG from multiple people, and then hope that one had a useful Ab to assist the ailing patient. This approach did not require any knowledge of the target cells nor even of the specificity of the acting Ab.

Fundamentally Ab are protein complexes that have the capacity to bind to receptors, generally other proteins, and in so doing facilitate immune system response. Simply stated; one end of the Ab binds to an immune cell while the other end binds to a specific antigen, Ag, that is expressed on another cell. This Ag is a foreign molecule and the binding of the two, immune cell and antigen, sets off an immune response that leads to the destruction of the invading party.

Monoclonal Ab have been available for decades and they currently can be engineered to target specific Ag that are present in specific diseases. These monoclonal Ab are fundamentally a normal Ab, such as IgG, which was generated from some Ag presentation in a murine environment or some other similar environment. Structurally it is a regular Ab and the only difference is that it was made for a specific Ag.

Now one can pose the question: if we can generate an Ab structure which can bind to specific proteins, can we engineer the structure in such a fashion so that it can bind to multiple Ag like molecules at the same time? Well it turns out that one can take two cells, each producing a different Ab and put them in a growth medium that contains polyethylene glycol. What can happen is cell fusion, namely the two cells become one and the result is that the Ab produced are some combination of the original two, a mix and match if you will. If you then take these hybrid Ab and use a separation column, a chromatographic column, where the fixed packing contains Ag for the desired Ab structure, then filtering it was are left with the new Ab at high concentration. Namely we have for example a bispecific antibody, an Ab which binds to two desired Ag.

We can take this a step further and get tri-specific antibodies and the list goes on.

As Fan et al note:

Bispecific antibodies (BsAbs) recognize two different epitopes. This dual specificity opens up a wide range of applications, including redirecting T cells to tumor cells, blocking two different signaling pathways simultaneously, dual targeting of different disease mediators, and delivering payloads to targeted sites. The approval of catumaxomab (anti-EpCAM and anti-CD3) and blinatumomab (anti-CD19 and anti-CD3) has become a major milestone in the development of bsAbs. Currently, more than 60 different bsAb formats exist, some of them making their way into the clinical pipeline. This review summarizes diverse formats of bsAbs and their clinical applications and sheds light on strategies to optimize the design of bsAbs….

BsAbs can not only bridge therapeutics (e.g., T cells, drugs) and targets (e.g., tumor) but also simultaneously block two different pathogenic mediators . In the near future, bsAbs might improve treatment options against cancer, autoimmune diseases, and inflammatory diseases. Two bsAbs have been approved with an impressive treatment profile. The success of bsAbs has captured the attention of pharmaceutical companies, with different companies devising new formats. Success aside, several critical hurdles remain, as only few formats have successfully moved into clinical trials. Large-scale production and purity are long-term pursuits.

The ideal platform should encompass the entire development process from discovery and preclinical studies to clinical material production, to allow rapid discovery of potent lead bsAbs and purification of clinical-grade bsAbs in a short time. Thus, simplifying the structure and production procedure and utilizing a powerful production platform are the keys when designing a bsAb format. The identification of target pairs and bsAbs with potential synergistic effects also poses a big challenge, necessitating a high-throughput approach.

Moreover, immunogenicity is a complex issue in drug design and development. In clinical trials, adverse effects are often reported and hamper the success of bsAbs. For example, toxicity of the bispecific 4G7 × H22 leads to the termination of its clinical study. Most adverse effects are mainly caused by a "cytokine storm." With the development of bsAbs, there is hope for the availability and approval of more therapeutic alternatives in future.

As Runcie et al note:

Polyspecific monoclonal antibodies (PsMabs) are genetically engineered proteins that can simultaneously engage two or more different types of epitopes. They show several advantages over monoclonal antibodies in that they can:

1) redirect specific polyclonal immune cells such as T cells and NK cells to tumor cells to enhance tumor killing,

2) simultaneously block two different pathways with unique or overlapping functions in pathogenesis,

3) potentially increase binding specificity by interacting with two different cell surface antigens instead of one, and

4) reduce cost in terms of development and production when compared to multiple single based antibodies used in combination therapy or compared to the production of CAR-T cells.

Runcie et al summarize these in the following Table (as modified)

Advantages	*Disadvantages*
Amenable for large scale production	Hetero-dimerization of chains may make the molecule inefficient; early methods had low production yields
More efficient binding to target	Steric inhibition of engaging sites
Able to engage T cell or NK cells (MHC agnostic) by a cell combining site	Potential antigenic cytokine release syndrome
Stability	Small molecules can be rapidly cleared; larger ones may aggregate; potential immunogenicity
Not patient specific; target specific	Tight white cell binding may change bio-distribution
Can be a carrier of radioisotope or chemotherapy	Potential poor internalization of molecule if combined with cytotoxic agent
Can be used for imaging	Need for external epitope
Can serve as an immune enhancer	Affinity for target epitope and effector cell critical
Can be encapsulated in a liposome	Large molecules have less intra-tumoral penetration.
Can be combined with other immunological agents	May enhance toxicity if combined with classical immunological agents
Bystander effect	

The logic behind polyAb is somewhat simple:

1. The immune system has the capability to attack invading entities, cells, viruses, etc.

2. However, sometimes these entities may hide or multiply at such a rapid rate to cause massive problems before the immune system can adequately respond.

3. Antibodies have been shown to be a powerful entity to identify and assist in attacking these harmful invaders.

4. However there are times when the immune system needs a jump start either as a vaccine or as an immunotherapeutic agent.

5. This has generally been accomplished via monoclonal antibodies, MAb, which help facilitate immune responses.

6. However many single targeted MAbs work briefly yet fail due to some secondary or even tertiary pathway where the invader can find an alternative path.

7. Some solutions may entail a cocktail of multiple MAbs.

8. The alternative is a polyAb which is a single molecule, protein structure, that can address the immune interface and at the same time multiple invader surface molecules allowing for both targeting and suppression.

Our approach herein is thus simply:

1. Review the immune system and the impact of antibodies

2. Review the MAb approaches including their generation

3. Review what is known about bi-specifics, which are the first of our polyAbs.

4. Provide a precis on tri-specifics, the next extension of a bi-specific but now addressing three Ag interfaces

5. Examine the production issues. Just because it works in one mouse does not mean we can treat millions of humans!

6. Provide an overview of several key applications in humans

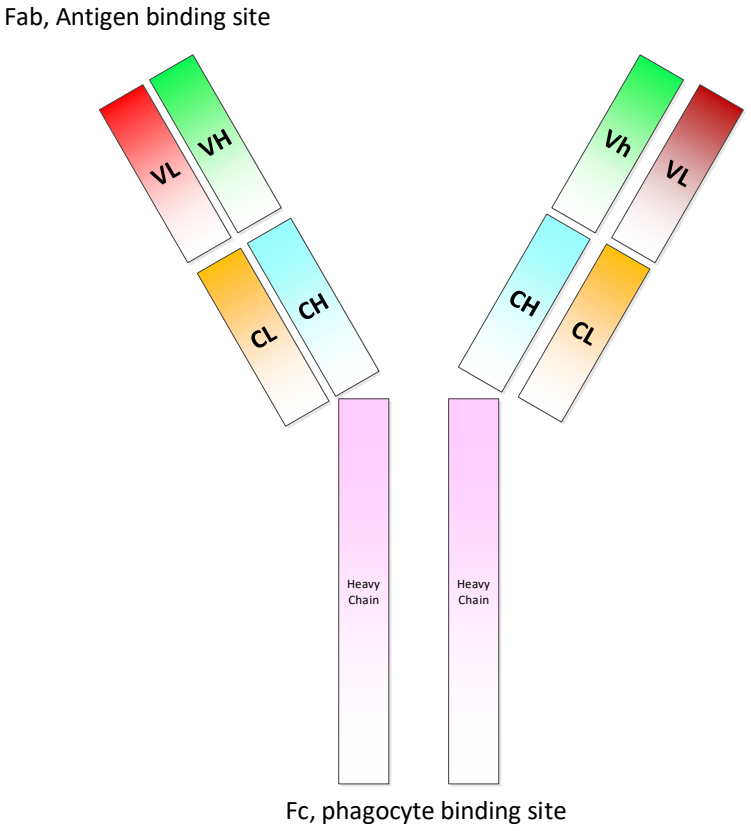
We end with some general observations as to the long term efficacy of polyAbs.

2 ANTIBODY BASICS

Antibodies are the product of the adaptive immune system using the B cells in concert with a variety of other immune system cells when presented with an antigen[1]. Thus the Ab-Ag complex is a means whereby infections can be mitigate against in a timely manner. The invader presents an Ag and if the individual has developed some form of immunity by prior presentation the invader is almost immediately attacked and eliminated by the Ab attaching to the invader on one end and to the attacking immune cells on the other end.

2.1 BASIC ANTIBODY

There are several forms of Ab but our primary focus in IgG. We show a classic IgG below. Note that it is a symmetric protein complex with the antigen binding element at one end and a site for phagocyte binding at the other. Once this binding is complete, in many circumstances, the immune system then attacks the invader. Fundamentally we have one invader and on phagocyte.



As Abbas et al note:

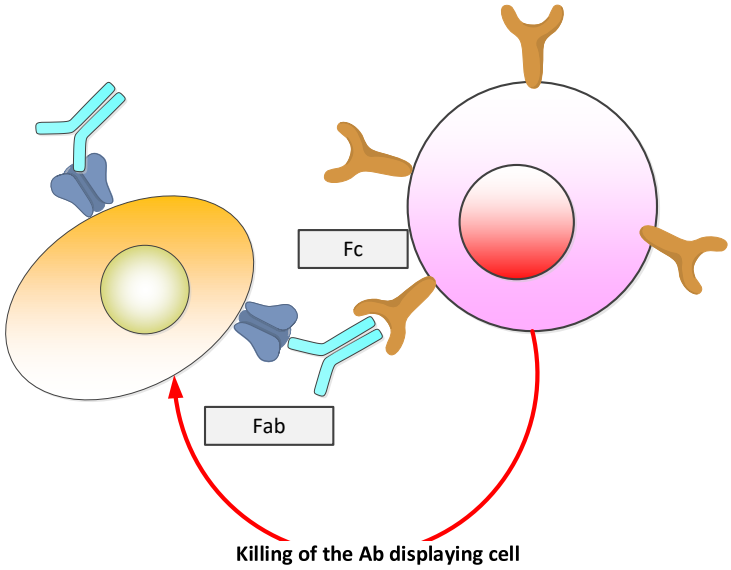
[1] See https://www.researchgate.net/publication/314090163_Cancer_Immunotherapy_A_Systems_Approach

Antibodies use their antigen-binding (Fab) regions to bind to and block the harmful effects of microbes and toxins, and they use their Fc regions to activate diverse effector mechanisms that eliminate these microbes and toxins. This spatial segregation of the antigen recognition and effector functions of antibody molecules was introduced. Antibodies sterically block the infectivity of microbes and the injurious effects of microbial toxins simply by binding to the microbes and toxins, using only their Fab regions to do so. Other functions of antibodies require the participation of various components of host defense, such as phagocytes and the complement system.

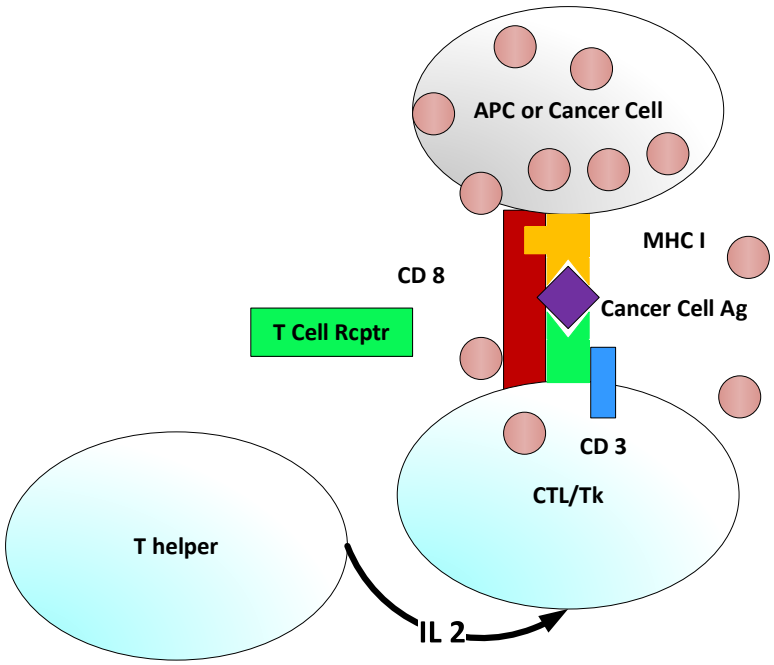
The Fc portions of immunoglobulin (Ig) molecules, made up of the heavy-chain constant regions, contain the binding sites for Fc receptors on phagocytes and for complement proteins. The binding of antibodies to Fc and complement receptors occurs only after several Ig molecules recognize and become attached to a microbe or microbial antigen. Therefore, even the Fc-dependent functions of antibodies require antigen recognition by the Fab regions. This feature of antibodies ensures that they activate effector mechanisms only when needed, that is, when they recognize their target antigens.

Fc Receptor	Affinity for Ig	Cell Distribution	Function
FcyRI (CD64)	High (Kd ~10^{-9} M); binds IgG 1 and lgG3; can bind monomeric IgG	Macrophages, neutrophils; also eosinophils	Phagocytosis; activation of phagocytes
FcyRIIA (CD32)	Low (K_d -‧‧‧0.6 2.5x1 O'^{6} M)	Macrophages, neutrophils; eosinophils, platelets	Phagocytosis; cell activation (inefficient)
FcyRIIB (CD32)	Low (K_d -0.6-2.5x1 O'^{6} M)	B lymphocytes, DCs, mast cells, neutrophils, macrophages	Feedback inhibition of B cells, attenuation of inflammation
FcyRIIIA (CD16)	Low (K_d -0.6-2.5x10'^{6} M)	NK cells	Antibody-dependent cellular cytotoxicity (ADCC)
FceRI	High (Kd~10‧10M); binds monomeric IgE	Mast cells, basophils, eosinophils	Activation (degranulation) of mast and basophils

The following Figure depicts the basis paradigm. The Fc end attached to a TCR and the Fab to the invader with the antigen. One that is complete, barring other possible inhibitory blocks then the T cell releases cytokines or other cell killing agents to rid the body of the invader.



We start with a brief overview of antibody basics. There is a significant body of literature on this topic so we will only outline what is essential to understand the functions of a polyAb. It is important to understand that even with an Ag attachment there may be other inhibitory paths that prevent the elimination. We depict some of these below. We have the Thelper and the CTL, or cytotoxic T lymphocyte activated when attached to a cell with an Ag. However there is always the "self" and "nonself" issue with inhibitory paths.



We shall proceed to discuss some of these multiple paths. It is often due to these paths that a single Ab connection may be necessary but not sufficient.

2.2 T CELL MECHANICS

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

From Freeman, we have an excellent summary description:

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

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

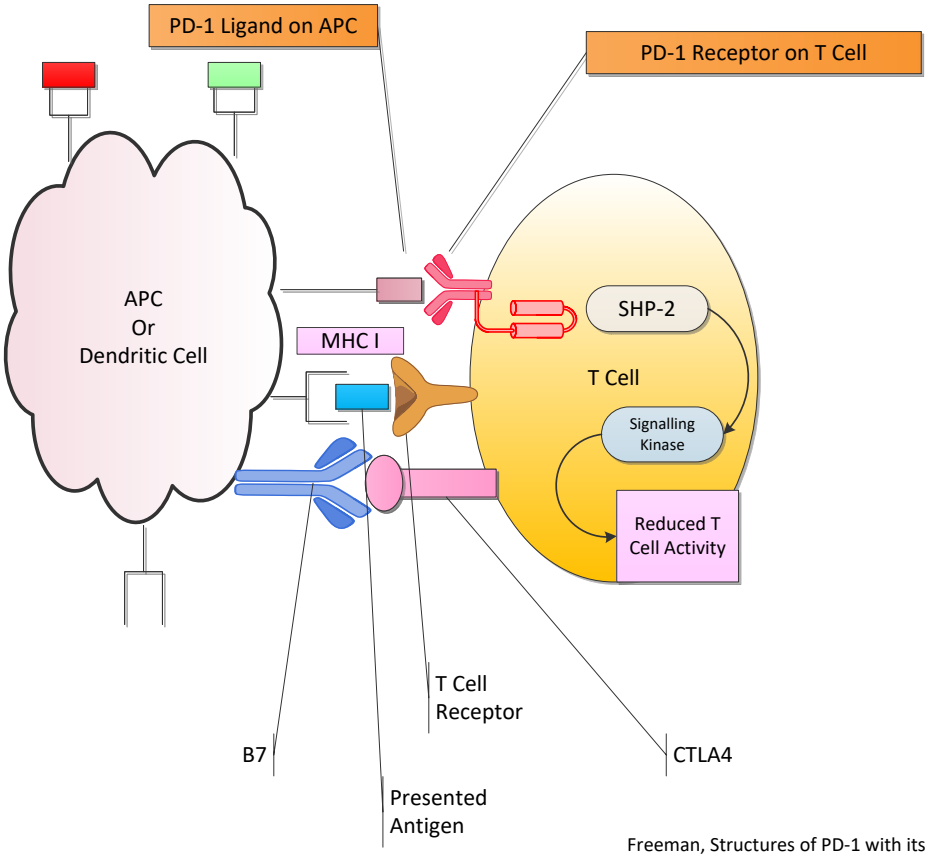
The threshold for the initiation of an immune response is set very high, with a requirement for both antigen recognition and costimulatory signals from innate immune recognition of ‘‘danger’’ signals. Paradoxically, T cell activation also induces expression of coinhibitory receptors such as programmed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

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

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

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

This process is shown graphically below. All three elements are shown; activator, inhibitor, and pathway. What is not shown are the multiplicity effects. This is a classic example of why a single path may not be sufficient.



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

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

3 MAB

Antibodies are powerful molecules developed as part of the adaptive immune system. On the one hand, once activated and generated in volume by the B cells, they set out and attach themselves to the antigens on the targeted cells and the Complement system of the Innate immune system takes over and kills the cell. Thus, when we use antigens for vaccines we are essentially priming the Adaptive system to have a large number of Abs ready and able to attack if necessary. On the other hand, recent therapeutics use antibodies to essentially attack certain receptors on cancer cells so that they may subsequently be attacked by a normal immune process. As we had noted in our discussion of the NK cells and of Set Points, there are also Check Points which will stop a normal immune attack, especially on cancer cells. Thus, the approach is to use an Ab to block the blocker, and then allow the immune system to do its job. In this Chapter, we examine how to make specific Abs.

Monoclonal antibodies, Mabs, have been available for decades[2]. Initially they were murine in development but over the past decade we have seen the development of hundreds of new Mabs for a variety of disorders. We will examine them here but we will do so in a manner which constructs another approach to the engineering of immune systems. The work was noted by Nobel Prizes in 1984[3].

We first review some of the elements of the immune system as relates to antibodies and then discuss the Mab development and evolution and finally examine how Mabs have been developed for various disorders.

To begin simply, a MAb is a plain vanilla antibody, IgG, which on the Fab end is matched to a specific antigen, Ag, and on the Fc end to a specific immune cell. The challenge is to determine the Ag. As we shall see, the Ag must reflect a cell whose presence is desired to be attacked and only that cell type. If the Ag is too broad we may get some type of cytokine storm. If it is too specific, than any alteration of the targeted Ag in vivo may inhibit the targeting. In addition the source of the IgG backbone, say a mouse, must not add additional Ag targets independent of what is sought after to eliminate. This MAb design is complex and of course costly.

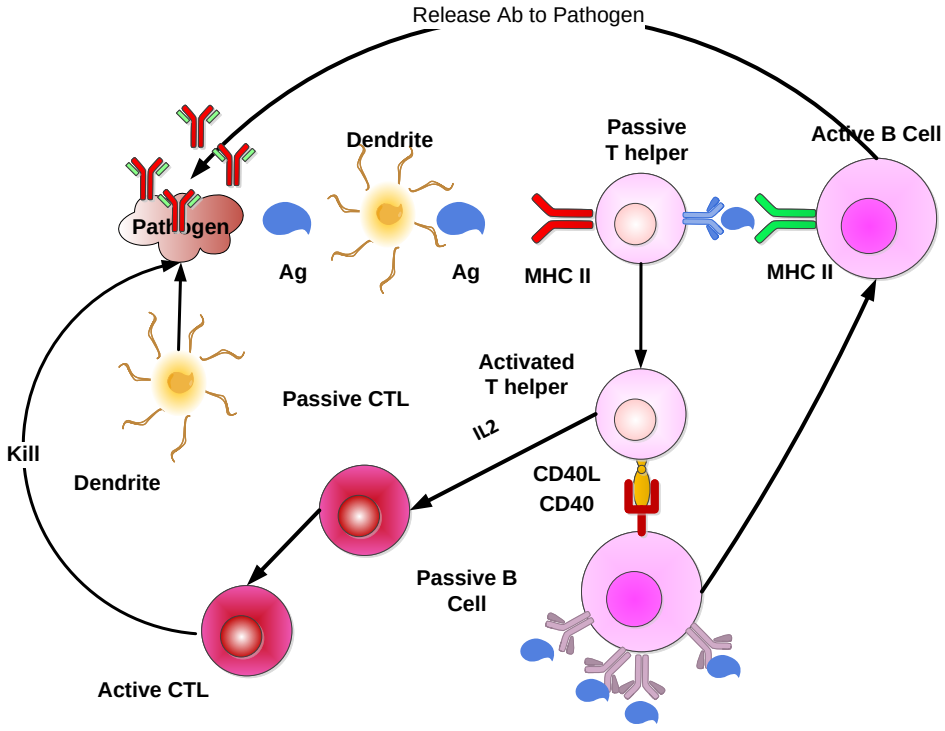
We proceed in this section to examine the MAb targeting, design, and production. This is done not as an end in itself but as a stepping stone for poly-Ab

[2] See Nature Immunology for some historical context. http://www.nature.com/milestones/mileantibodies/Milestones_Poster.pdf and http://www.nature.com/milestones/mileantibodies/collection/index.html Also see Marks, L, The Lock and Key of Medicine: Monoclonal Antibodies and the Transformation of Healthcare, Yale University Press; 1 edition (June 30, 2015).

[3] See Nobel lectures by Milstein and Kohler.

3.1 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.



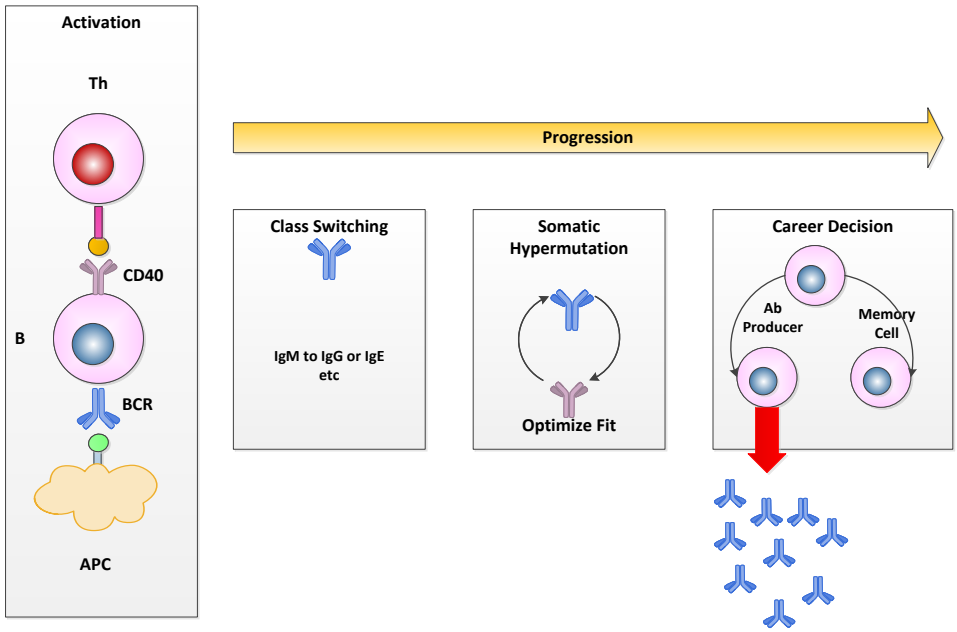
Recall that interaction of antibody with antigen initiates the classical pathway of complement activation. This biochemical cascade of enzymes and protein fragments facilitates destruction of microbes by the membrane attack complex (MAC), by increased opsonization through C3b binding of microbial surfaces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

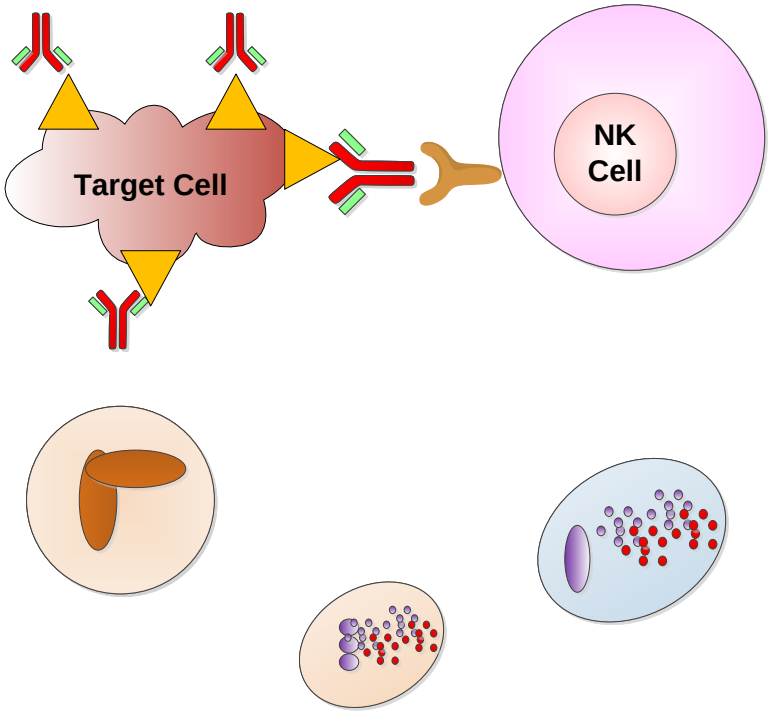
Upon binding to antibody, C1q undergoes a conformational change that leads to the sequential binding and activation of the serine proteases C1r and CIs. The C1qrs complex has enzymatic activity for both C4 and C2, indicated by a horizontal bar as either C1qrs or abbreviated as C15. Activation of C1qrs 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.

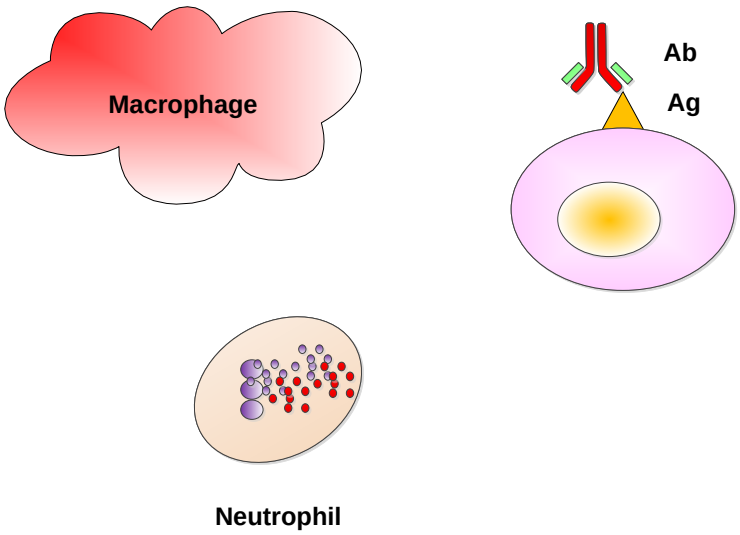
The antibody-dependent cell-mediated cytotoxicity can be described as follows. The “tagging" of an invasive organism can attract phagocytic cells and other cytolytic cells. FcRs on NK cells (FcyRIII) and eosinophils (FcyRI, FcbRI, and FcotRI) are IgG-, IgE-, and IgA-specific. The bound cells may be bacteria,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

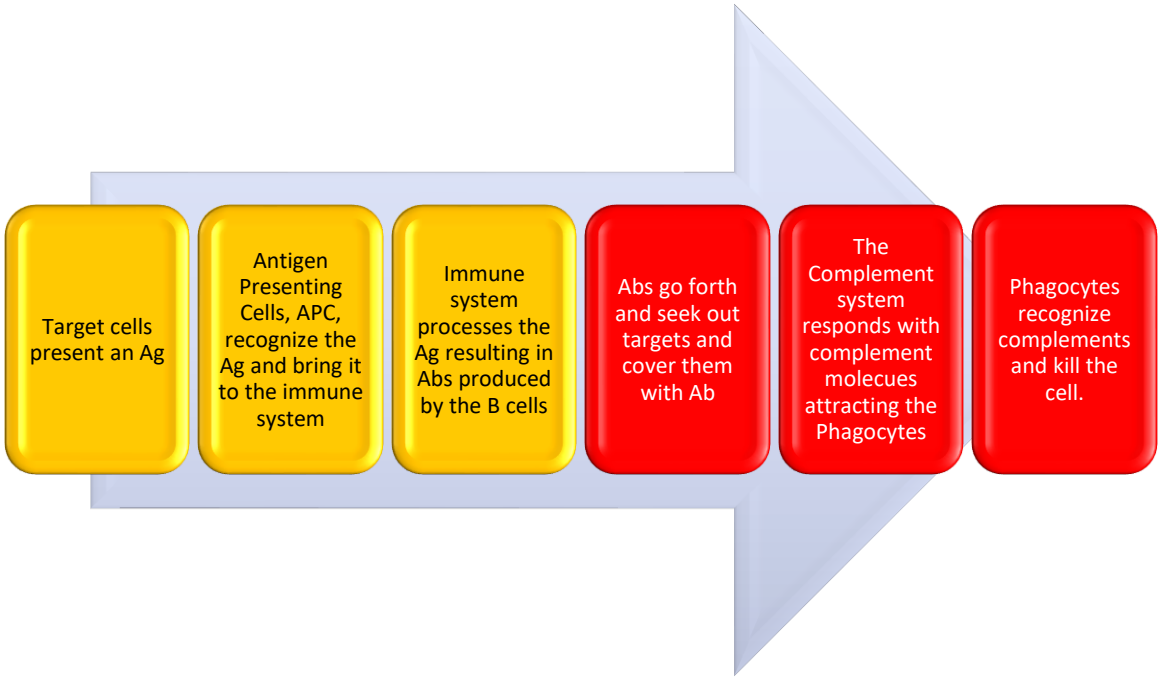
2. Antigen presenting cells see the Ag and carry it to the adaptive system.

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.

3.2 MAB DEVELOPMENT

Mab development has progressed from mouse models to genetically engineered human analog. It is now possible to accurately design a fully human Ab for use in therapeutic applications. Details are provided in such works as those by Steinitz.

From Steinitz we have:

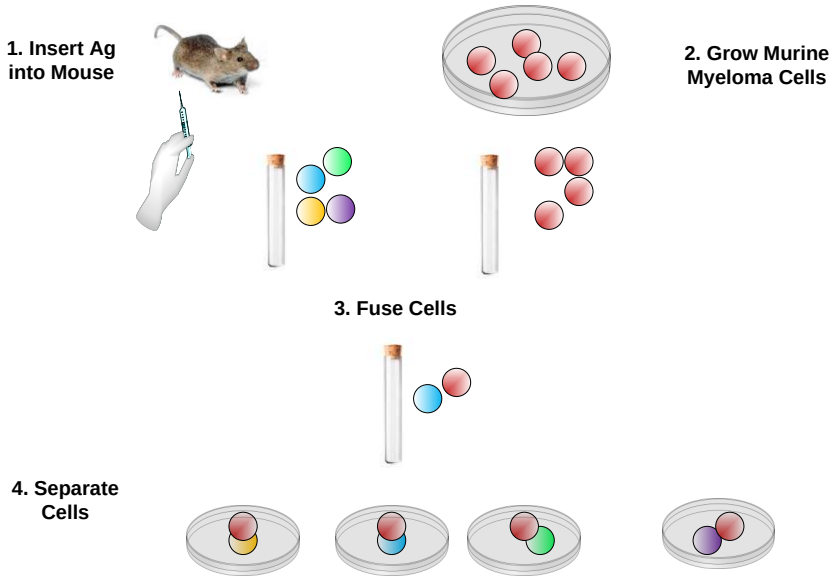
Human antibodies are elicited in response to invading substances (antigens) by B cells. The antigen(s) could be a part of an invading microbe, nonself-cells, or mutated/altered self-cells such as cancer cells.

For a complete immune response various immune cells, in addition to B cells, function together to activate the overall immune system. As a result of the immune response B cells produce antibodies that are specific to an antigen or part (epitope) of an antigen. Antibodies by themselves can destroy or inactivate cells and neutralize substances via a number of mechanisms mediated by nonbinding regions of the antibody.

These mechanisms may require complement and other immune cells, such as NK cells. Because of therapeutic and diagnostic applications of antibodies in human health (control of infectious diseases, autoimmunity, cancer, and other human ailments), they have played a central role in

investigative efforts to exploit them to their fullest extent. The first mAbs, of murine origin, were developed more than 35 years ago, as an unlimited source of a single specificity.

However, once in the clinic the xenogeneic nature of the murine mAb resulted in a human anti-murine antibody (HAMA) response in patients that negated the effects of the therapy. Due to these unwanted HAMA responses, various modifications of mAbs to reduce or eliminate the undesired side effects in human were developed which led to the development of chimerized, humanized, and totally human versions. In addition, innovative in vivo diagnostic and therapeutic applications led to modifications of antibody size [single chain (sFv)] and enhancement of their biological activities



Monoclonal antibodies are created by injecting human cancer cells, or proteins from cancer cells, into mice. The mouse immune systems respond by creating antibodies against these foreign antigens. The murine cells producing the antibodies are then removed and fused with laboratory-grown cells to create hybrid cells called hybridomas. Hybridomas can indefinitely produce large quantities of these pure antibodies. Monoclonal antibodies can be developed to act against cell growth factors, thus blocking cancer cell growth. Monoclonal antibodies can be conjugated or linked to anticancer drugs, radioisotopes, other biologic response modifiers, or other toxins. When the antibodies bind with antigen-bearing cells, they deliver their load of toxin directly to the tumor. Monoclonal antibodies may also be used to preferentially select normal stem cells from bone marrow or blood in preparation for a hematopoietic stem cell transplant in patients with cancer. Monoclonal antibodies achieve their therapeutic effect through multiple direct and indirect mechanisms

1. Can have direct effects in producing apoptosis or programmed cell death.

2. Can block growth factor receptors, effectively arresting proliferation of tumor cells.

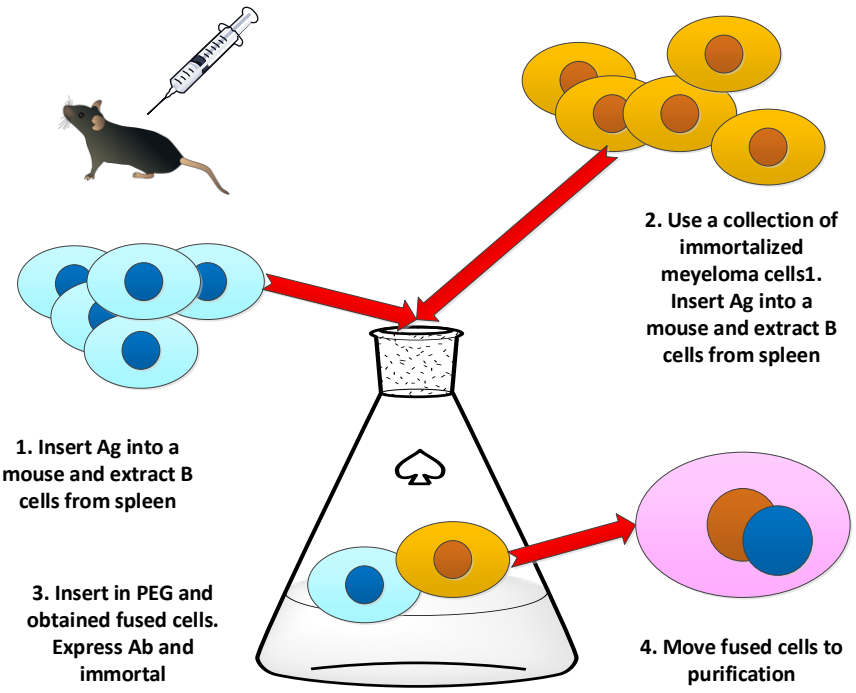
3. Can bring about anti-idiotype antibody formation in cells that express monoclonal antibodies.

4. Recruiting cells that have cytotoxicity, such as monocytes and macrophages. This type of antibody-mediated cell kill is called antibody-dependent cell mediated cytotoxicity (ADCC),

5. Also bind complement, leading to direct cell toxicity, known as complement dependent cytotoxicity (CDC).

Now we will examine the process in some more detail. As will be seen the process is complex and does have possible points for ineffective production. Scaling up this process is often costly and demands significant quality assurance.

The Figure below depicts the first set of steps.

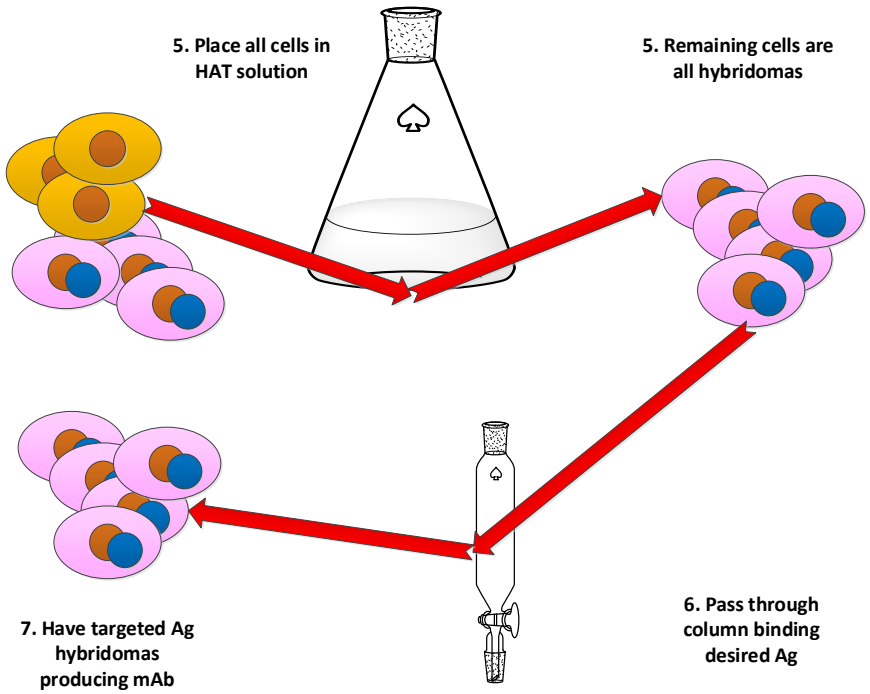


Let us go through the steps.

1. We first need a generating entity which can produce an Ab from a known Ag. Note first we must have the Ag well defined and that the Ag will be what is expected in the natural setting. An extracted Ag may suffer deformities and the resulting Ab may be generated but only to meet the deformed Ag. Thus a careful check and balance at this step is essential. Also the entity used to generate the Ab is a crucial choice. Here we show a mouse for a murine Ab. Again as we noted above a murine Ab has certain advantages and disadvantages.

2. We the need immortalized cells to act as a growing medium. We show immortalized myeloma cells. There are other possible immortalized cells as well such as HET 293[4].

3. We then add the two cell lines to a solution of polyethylene glycol, PEG, and slowly circulate the cells in the solution for some period of time. The net result is that several of the cells are fused. There may still be unfused cells and we must remove them.



4. The placement of the cells in a HAT solution kills off the myeloma cells. In the classic paper by Grimaldi and French they note how to accomplish this task:

Myeloma cell lines can be selected in a medium containing hypoxanthine, thymidine, and aminopterin (HAT), thus nonfused myeloma cells die and only those cells fused to normal cells survive. Myeloma fusion partners are deficient in an enzyme required for the salvage pathway of nucleotide synthesis. These cells will die in HAT-containing medium because aminopterin blocks normal nucleotide synthesis and the enzyme deficiency blocks utilization of hypoxanthine or thymidine in the salvage pathway.

If myeloma and normal cells fuse, the resulting hybridoma will live indefinitely in culture because the normal cell supplies the missing enzyme for selection in HAT-containing medium and the myeloma cell immortalizes the cell line. Unfused normal lymphocytes will only survive in tissue culture for approximately 1 week before they die. The choice of a myeloma cell fusion

[4] See Lin et al as well as Kim et al.

partner should be genetically compatible with the immunized B-cell source because hybridomas generated from cells of the same species are more stable than hybridomas generated from different species.

5. Finally we can take the remaining cells, extract and purify the IgG through a column where the fixed phase binds to the desired IgG.

3.3 CELL FUSION

There are four means for achieving cell fusion. Namely:

(i) electrical cell fusion wherein an electrical shock is applied to the cells and this facilitates the opening of the cell membrane and facilitates fusion.

(ii) PEG or polyethylene glycol cell fusion which has been a standard means. PEG chemically facilitates the wall opening and ultimate fusion. This technique has some issue since it also results in complex multifusions.

(iii) Sendai virus induced cell fusion wherein a complex process of using the viral cell and its inclusion in the target cell sets of a process amenable to cell-cell fusion.

(iv) optically controlled thermoplasmonics which uses an infrared heating effect to "open" cells and facilitate fusion

There are some interesting issues regarding cell fusion. In the case of MAbs we have two cells fusing. namely the B cell with the Ab and the myeloma immortalized cell. Both cells have separate nuclei and nucleoli. There is extensive work on fusion and the resultant structure of the cell. Namely do we get two nuclei or one and if one are there two nucleoli or one? Moreover the control of the cell cycle is complex and we have examined elsewhere as well.

From Oren-Suissa and Podbilewicz we have:

Researchers have identified new paradigms of cell fusion based on what we have learned from enveloped viruses and intracellular membrane fusion processes. Different classes of enveloped viruses fuse to their target cells and can also fuse cells expressing their specialized membrane glycoproteins that induce membrane fusion . Stage III of the cell-fusion process summarizes the current working model of how FF proteins (EFF-1 and AFF-1) from C. elegans fuse cell membranes resulting in cytoplasmic mixing and reorganization of the cytoskeleton…

NON-SELF FUSION (TYPE I) Examples of this type of cell–cell fusion include most types of sex from yeast to humans, with the notable exception of conjugation in bacteria. It was hypothesized by Tatum and Lederberg that bacterial conjugation will involve cell fusion ‘‘we postulate a sexual phase in this strain of E. coli: a cell fusion which allows the segregation of genes in new combinations into a single cell’’.

However, it has been established that type IV secretion and the formation of a proteinaceous tube connecting conjugating bacteria is the mechanism responsible for conjugation in E. coli. Sexual fusion in protists, fungi, plants, and animals is a heterotypic cell–cell fusion process in which cells of different sexes or mating types unite their membranes to share and combine their genetic materials. Currently, there are a number of systems in which non-self-cell-fusion pathways have been studied and different components required for sexual fusion have been identified …

Monoclonal antibodies are produced by hybrid cells obtained by fusing a cancerous myeloma cell with a B lymphocyte from the spleen of animals immunized with a specific antigen. Hybridomas producing monoclonal antibodies are then selected in vitro. The first hybridomas in the early 1970s were between rat and mouse cells.

The selection and screening of fused cells that divide and produce high amounts of specific antibodies was a major breakthrough, and the Nobel prize was awarded to Kohler and Milstein . Fusion in a lab dish can be obtained using polyethylene glycol (PEG), electric fields, lasers, or viral fusogens such as Sendai viruses. Researchers have induced self and non-self-cell fusion in tissue culture, in whole C. elegans embryos, and between diploid differentiated cells and enucleated oocytes to address many diverse biological questions ranging from the cell cycle to intracellular trafficking and animal cloning. Thus, under the right conditions PEG, viral and FF fusogens induce cell–cell fusion .

From Hernández and Podbilewicz we have:

Cell-cell fusion is essential for fertilization and organ development. Dedicated proteins known as fusogens are responsible for mediating membrane fusion. However, until recently, these proteins either remained unidentified or were poorly understood at the mechanistic level. Here, we review how fusogens surmount multiple energy barriers to mediate cell-cell fusion. We describe how early preparatory steps bring membranes to a distance of ~10 nm, while fusogens act in the final approach between membranes. The mechanical force exerted by cell fusogens and the accompanying lipidic rearrangements constitute the hallmarks of cell-cell fusion. Finally, we discuss the relationship between viral and eukaryotic fusogens, highlight a classification scheme regrouping a superfamily of fusogens called Fusexins, and propose new questions and avenues of enquiry.

The above authors then proceed to examine in some detail the various cell-cell fusion modalities. For example we have (i) virus-host fusion as is seen in COVID-19, (ii) gamete fusion as seen in reproduction, and (iii) somatic cell fusion as we examine herein.

Now cell-cell fusion is common but nucleus-nucleus fusion has a significant roles as well. As Spees et al have noted:

To investigate stem cell differentiation in response to tissue injury, human mesenchymal stem cells (hMSCs) were cocultured with heatshocked small airway epithelial cells. A subset of the hMSCs rapidly differentiated into epithelium-like cells, and they restored the epithelial monolayer. Immunocytochemistry and microarray analyses demonstrated that the cells

expressed many genes characteristic of normal small airway epithelial cells. Some hMSCs differentiated directly after incorporation into the epithelial monolayer but other hMSCs fused with epithelial cells. Surprisingly, cell fusion was a frequent rather than rare event, in that up to 1% of the hMSCs added to the coculture system were recovered as binucleated cells expressing an epithelial surface epitope. Some of the fused cells also underwent nuclear fusion

The slight mention is important. Cell-cell fusion we can accomplish in a variety of ways yet the next step of nucleus-nucleus fusion still demands examination. The one must progress to the nucleolus level as well.

Alvarez-Dolado presents an interesting set of paradigms which include nucleus fusion as well. He notes as follows:

Cell Fusion Products.

A. Cells of the same lineage fuse to form a giant cell with multiple nuclei, known as syncytium. Skeletal muscle and macrophages are examples of syncytia.

B. Cells of different lineage fuse to form a cell with multiple nuclei, called heterokaryon. The stable heterokaryon might acquire new properties, being able to proliferate and differentiate.

C. If a heterokaryon rearranges its multiple nuclei in a single nucleus we obtain a synkaryon. This process can take place by two different ways: deletion of supernumerary nuclei (upper panel), or by nuclear fusion and posterior reductive mitosis (lower panel). In the first case, fused cells mix their cytoplasm. This facilitates epigenetic modifications, what may lead to phenotype reversion obtaining a cell similar to the original one, or may lead to transdifferentiation originating a completely different cell type (pinkish colour). In the second case, nuclear fusion makes cells to mix DNA. This facilitates genetic reprogramming and acquisition of new phenotypes. It is important to note how, after all these processes (fusion, reprogramming, mitosis…), some of the final synkaryons are undistinguishable from the normal original cell types.

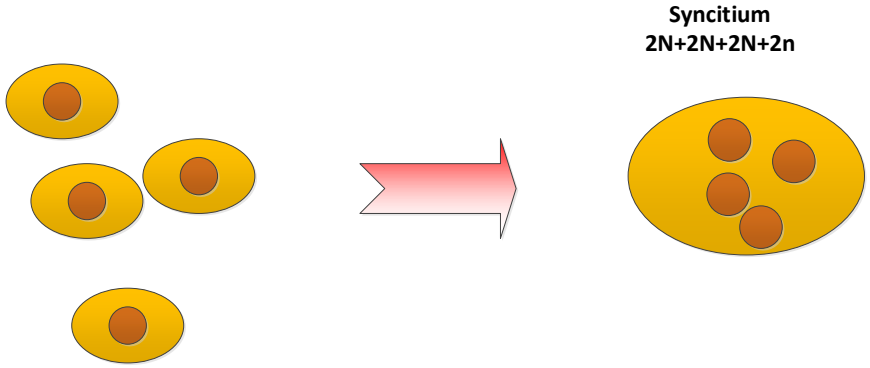
D. Cells of the same lineage might suffer fusion and posterior nuclear rearrangement to obtain a single 2n nucleus. The new synkaryon would be very difficult to distinguish and detect due its similitude to original cells. Consequently, fusion events might be undercover and underestimated.

E. Risks of cell fusion. Viral transfer is facilitated by cell fusion and posterior DNA recombination. After fusion, cellular and viral genomes are mixed suffering recombination in such a form that new virus might be able to infect new cell types.

We demonstrate some of these below:

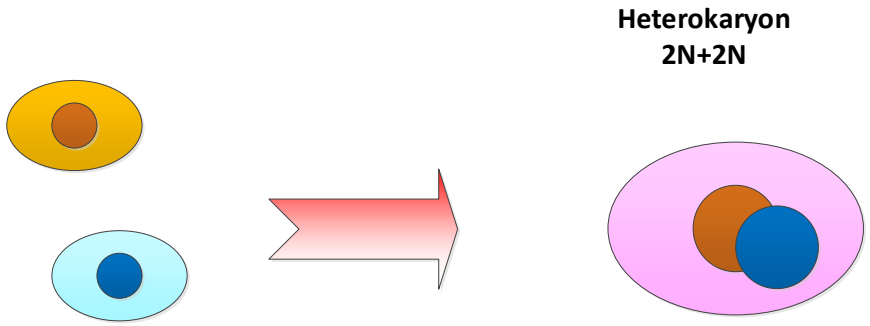
3.3.1 Common Cell Fusion

This is just a classic cell-cell fusion of the same type of cell. We frequently see these in some malignancies and in normal cells as well.



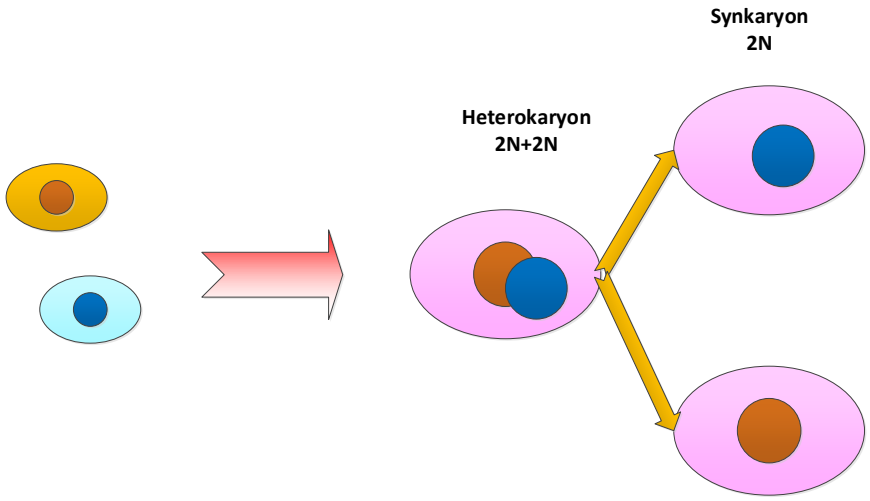
3.3.2 Disparate Cell Fusions

In this cell-cell fusion we obtain a single cell but with two nuclei.



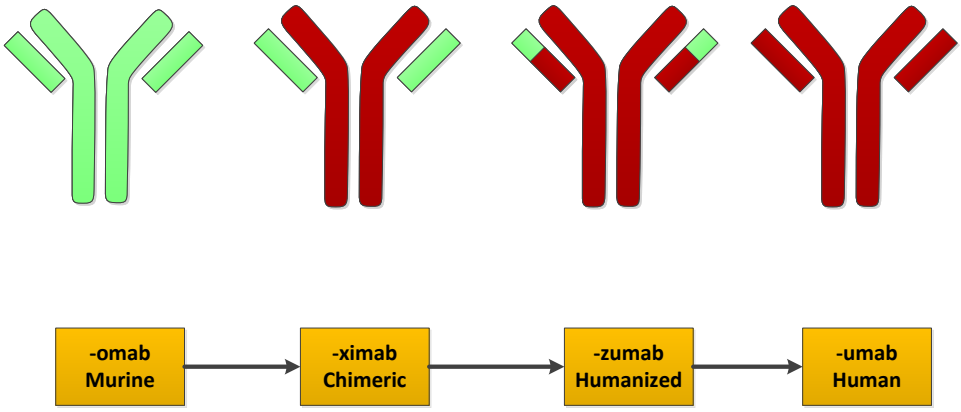
3.3.3 Synkaryon

This is a case where we have a cell-cell fusion and then a separation into two cells having different nuclei.



3.4 MAB TYPES

There is an evolution of Mab applications from those which were fully mouse generated which are murine to those fully human. The collection is shown below. Namely we have a murine, chimeric, humanized and human. Recall that the binding to the Ab occurs at the epitope site on one of the two arms[5].



In the current therapeutic market, most if not all are human genetically engineered and referred to as -umab.

Iborra et al have presented a summary paper on the multiple types of Ab implementation. They note the different sources as below:

[5] See Shepard et al

3.4.1 Murine Monoclonal Ab

Antibody-secreting hybridomas are derived from the fusion of a murine myeloma cell line that can grow indefinitely and B splenocytes from an immunised mouse. A large number of mouse plasmacytoma immortalised cell lines, secreting different immunoglobulin classes, are available in the American Type-Culture Collection (ATCC). Non-secretory myeloma cell lines deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) have been established as fusion partners, because they are not able to use the salvage pathway for DNA synthesis in the presence of aminopterin.

Cell fusion is facilitated by polyethylene glycol, by a viral infection, or by electroporation. To select viable hybridomas, cells are grown in selective medium with hypoxanthine, aminopterin and thymidine (HAT medium), in which only the hybridomas proliferate. The myeloma cells that fuse with another myeloma cell or do not fuse at all, die in HAT medium since they are HGPRT-negative. B cells and B cell hybrids also die because they are not able to grow indefinitely. Hybridomas resulting from cell fusion are a heterogeneous population, with a broad range of antibody specificities. After screening for specificity, hybridomas are cloned by limiting dilution, normally to obtain single secreting clones.

3.4.2 Human Monoclonal Ab

Human monoclonal antibodies and monoclonal antibodies from several species, other than mouse or rat, are difficult to generate because of lack of myeloma cell lines. B cells may be derived from different lymphoid tissues, such as spleen, tonsil, lymph nodes and also from peripheral blood mononuclear cells (PBMCs). Several strategies for human monoclonal antibody production have been applied: immortalisation of B cells by virus, human B lymphocyte fusion with a heterologous myeloma cell line (heterohybridoma), homologous myeloma cell line (human hybridomas) or use of recombinant DNA technology.

3.4.3 Transgenic Mice

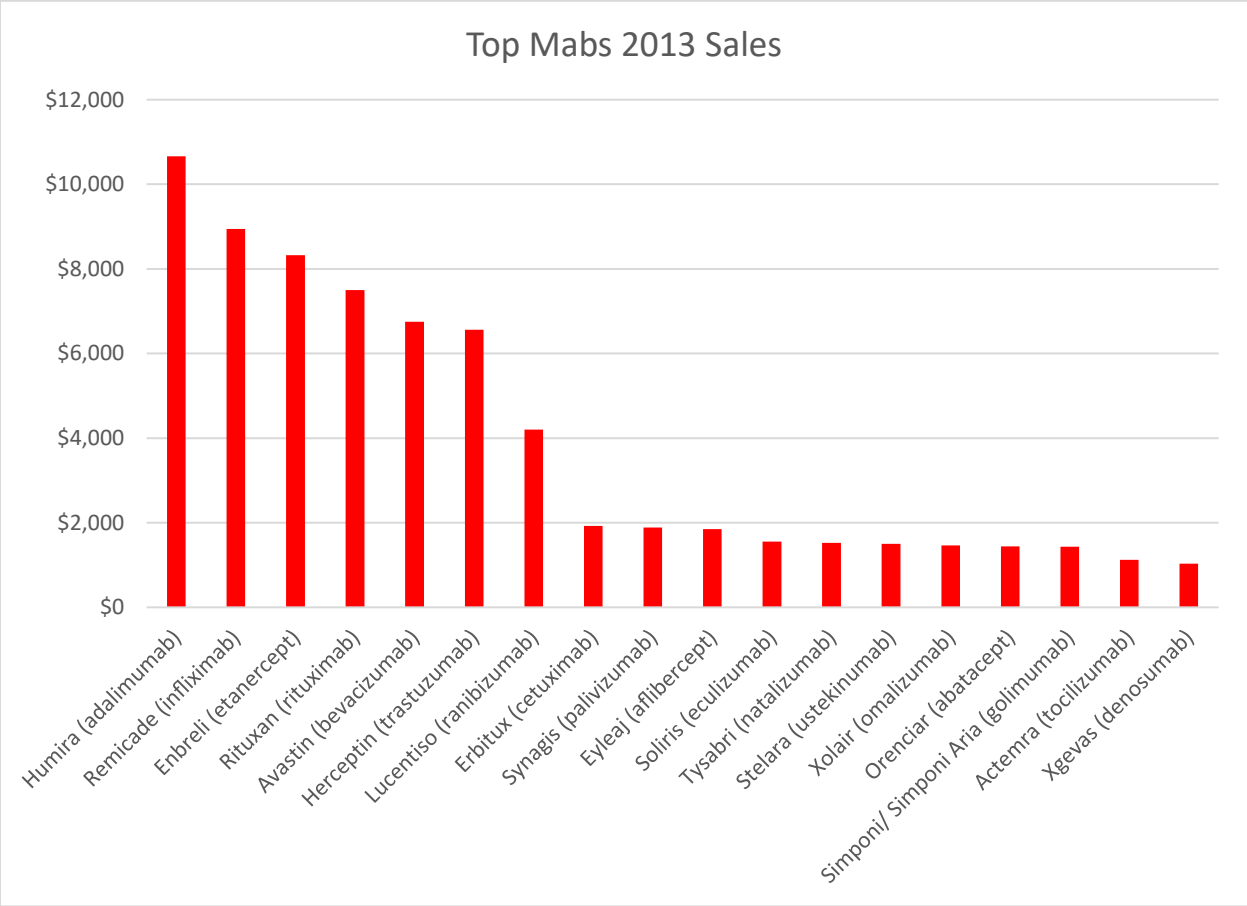
Transgenic mouse platform is another available technology to produce human monoclonal antibodies. Transgenic mice that express human antibody repertoires were first reported in 1994. Nowadays, more than 30 human monoclonal antibodies produced in transgenic mice are included in clinical trials. This approach maintains the advantage of mouse hybridoma technology for the production of monoclonal antibodies with high therapeutical potential uses

3.5 MAB APPLICATION

The following Table is, as modified, from Galluzzi et al and depicts many of the current Mabs and their applications.:

Therapeutic MAb	Disease	Date	Function
Alemtuzumab	Chronic lymphocytic leukemia	2001	Selective recognition/opsonization of CD52+ neoplastic cells
Bevacizumab	Colorectal carcinoma Glioblastoma multiforme Cervical carcinoma Lung carcinoma Renal cell carcinoma	2004	VEGFA neutralization
Brentuximab vedotin	Anaplastic large cell lymphoma Hodgkin's lymphoma	2011	Selective delivery of MMAE to CD30+ neoplastic cells
Blinatumumab	Acute lymphoblastic leukemia	2014	CD3- and CD19-specific BiTE
Catumaxomab	Malignant ascites in patients with EPCAM+ cancer	2009	CD3- and EPCAM-specific BiTE
Cetuximab	Head and neck cancer Colorectal carcinoma	2004	Inhibition of EGFR signaling
Denosumab	Breast carcinoma Prostate carcinoma Bone giant cell tumors	2011	Inhibition of RANKL signaling
Gemtuzumab ozogamicin	Acute myeloid leukemia	2000	Selective delivery of calicheamicin to CD33+ neoplastic cells
Ibritumomab tiuxetan	Non-Hodgkin lymphoma	2002	Selective delivery of 90Y or 111In to CD20+ neoplastic cells
Panitumumab	Colorectal carcinoma	2006	Inhibition of EGFR signaling
Pertuzumab	Breast carcinoma	2012	Inhibition of HER2 signaling
Obinutuzumab	Chronic lymphocytic leukemia	2013	Selective recognition/opsonization of CD20+ neoplastic cells
Ofatumumab	Chronic lymphocytic leukemia	2009	Selective recognition/opsonization of CD20+ neoplastic cells
Ramucirumab	Gastric or gastroesophageal junction adenocarcinoma	2014	Inhibition of KDR signaling
Rituximab	Chronic lymphocytic leukemia Non-Hodgkin lymphoma	1997	Selective recognition/opsonization of CD20+ neoplastic cells
Siltuximab	Multicentric Castleman's disease	2014	IL-6 neutralization
Tositumomab	Non-Hodgkin lymphoma	2003	Selective recognition/opsonization of, or selective delivery of 90Y or 111In to, CD20+ neoplastic cells
Trastuzumab	Breast carcinoma Gastric or gastroesophageal junction adenocarcinoma	1998	Selective recognition/opsonization of, or selective delivery of mertansine to, HER2+ cancer cells
Lenalidomide	Mantle cell lymphoma Myelodysplastic syndrome Multiple myeloma	2005	IKZF degradation and immunomodulation
Pomalidomide	Multiple myeloma	2013	IKZF degradation and immunomodulation
Thalidomide	Multiple myeloma	2006	IKZF degradation and immunomodulation
Trabectedin	Soft tissue sarcoma Ovarian carcinoma	2007	Reprogramming of tumor- associated macrophages

From the work of Ecker et al we have the following Table which complements the above:



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

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

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

3.6 Issues with Ab Approaches

There are a number of obstacles to successful therapy with monoclonal antibodies:

1. Antigen distribution of malignant cells is highly heterogeneous, so some cells may express tumor antigens, while others do not.

2. Antigen density can vary as well, with antigens expressed in concentrations too low for monoclonal antibodies to be effective.

3. Tumor blood flow is not always optimal. If monoclonal antibodies need to be delivered via the blood, it may be difficult to reliably get the therapy to the site.

4. High interstitial pressure within the tumor can prevent the passive monoclonal antibodies from binding.

5. Since monoclonal antibodies are derived from mouse cell lines, the possibility of an immune response to the antibodies exists. This response not only decreases the efficacy of monoclonal antibody therapy, but also eliminates the possibility of re-treatment.

6. Very rarely do we see cross-reactivity with normal tissue antigens—in general target antigens that are not cross reactive with normal tissue antigens are chosen. Despite these obstacles, there has been tremendous success in the clinical application of monoclonal antibodies in hematologic malignancies and solid tumors.

It should also be noted that Abs when sent out by the immune system basically attach to cells with Ag and then attract the Complement system to attack and destroy. However, when used to be a Checkpoint Inhibitor such as in PD-1 blockade, the attach to PD-1 yet do not activate the Complement system, they allow the immune system to attack in a different manner. Perhaps this is a difference in functioning or perhaps not. It has been noticed in many Mab trials that there are secondary effects, which frankly would be expected.

Baldo presents the following list of Mabs when he discusses their potential adverse responses.

Generic Name	Type of Mab	Target	Action	Approved	Trade Name
Catumaxomab	rat IgG2b / Mouse IgG2a bispecfic	epCaMc/CD3d	Binds both epCaM on tumor cell and CD3 on T cell	Malignant ascites	removab®
Ibritumomab tiuxetane	Murine IgG1κ	CD20	Binds B cells and kills with aDCC,f CDCf and radiatione	Non-Hodgkin lymphoma	Zevalin®
Tositumomab-131I	Murine IgG2aλ	CD20	Binds to and kills B cells with 131I	Non-Hodgkin lymphoma	Bexxar®
-ximabs					
Brentuximab vedoting	Chimeric IgG1κ	CD30h	antimitotic MMaeg	anaplastic large cell lymphoma; Hodgkin lymphoma	adcetris®
Cetuximab	Chimeric IgG1κ	eGFri	Binds to eGFr and turns off cell divisionj	Colorectal cancer; head and neck cancers	erbitux®
rituximab	Chimeric IgG1κ	CD20	Binds to CD20 on B cells leading to cell death	Non-Hodgkin lymphoma	MabThera® rituxan®
-zumabs					
alemtuzumab	Humanized IgG1κ	CD52k	eliminates lymphocytes	Chronic lymphocytic leukemia	Campath-1H®
Bevacizumab	Humanized IgG1κ	veGFl	angiogenesis inhibitor	Colorectal, lung, kidney, brain cancers	avastin®
Pertuzumab	Humanized IgG1κ	Her2m	Inhibits dimerization of Her2 with other Her receptors	Metastatic breast cancer	Perjeta®
Trastuzumab	Humanized IgG1κ	Her2	Prevents overexpression of Her2	Breast cancer	Herceptin®
Trastuzumab emtansinen	Humanized IgG1κ	Her2	mab-drug conjugate. as for trastuzumab plus cytotoxic effect of mertansine (DM1)o	advanced metastatic breast cancer	Kadcyla™
-umabs					
Denosumab	Human IgG2κ	raNKLp	Inhibits activation of osteoclasts by raNKL	Bone metastases; Giant cell tumor of the bone (GCTB)	Prolia® Xgeva®

Generic Name	Type of Mab	Target	Action	Approved	Trade Name
Ipilimumab	Human IgG1κ	CTLa-4q	Blocks interaction of CTLa-4 with its ligandsr and enhances T cell activation	Metastatic melanoma	yervoy®
Ofatumumab	Human IgG1κ	CD20	Binds to CD20 on B cell causing cell death	Chronic lymphocytic leukemia	arzerra®
Panitumumab	Human IgG2κ	eGFri	Binds to and prevents activation of eGFr	Colorectal cancer	vectibix®

4 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[6].

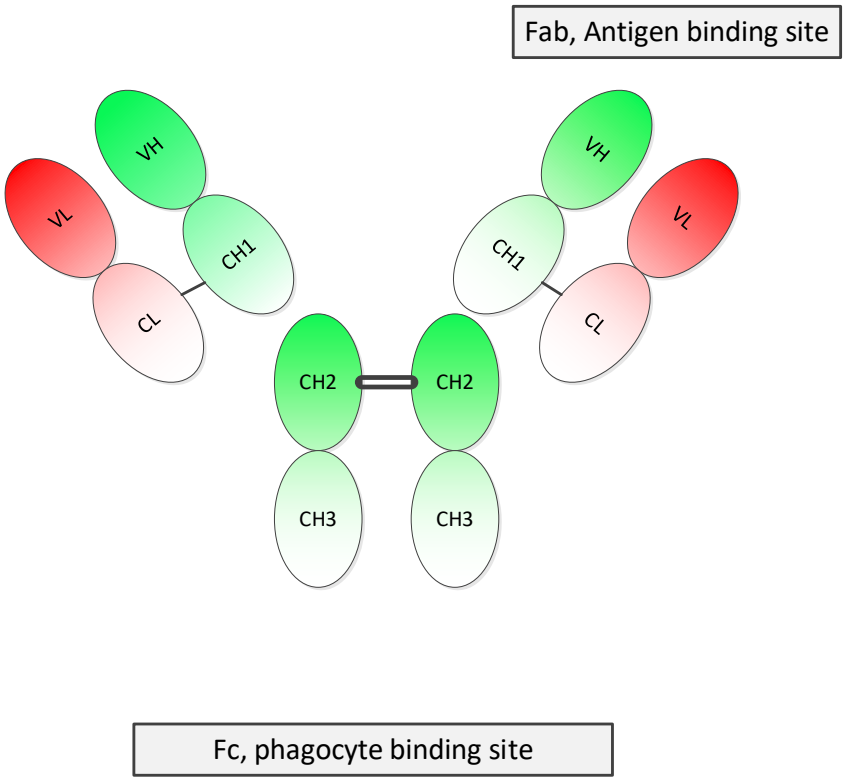
Other firms have split up the task by creating two bispecifics. One targets a tumor antigen and CD28 or another growth-signal receptor; the other binds to the tumor antigen plus CD3. "One of our hopes is that this costimulatory bispecific may help us unlock responses in solid tumors," says Lowy, whose 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.

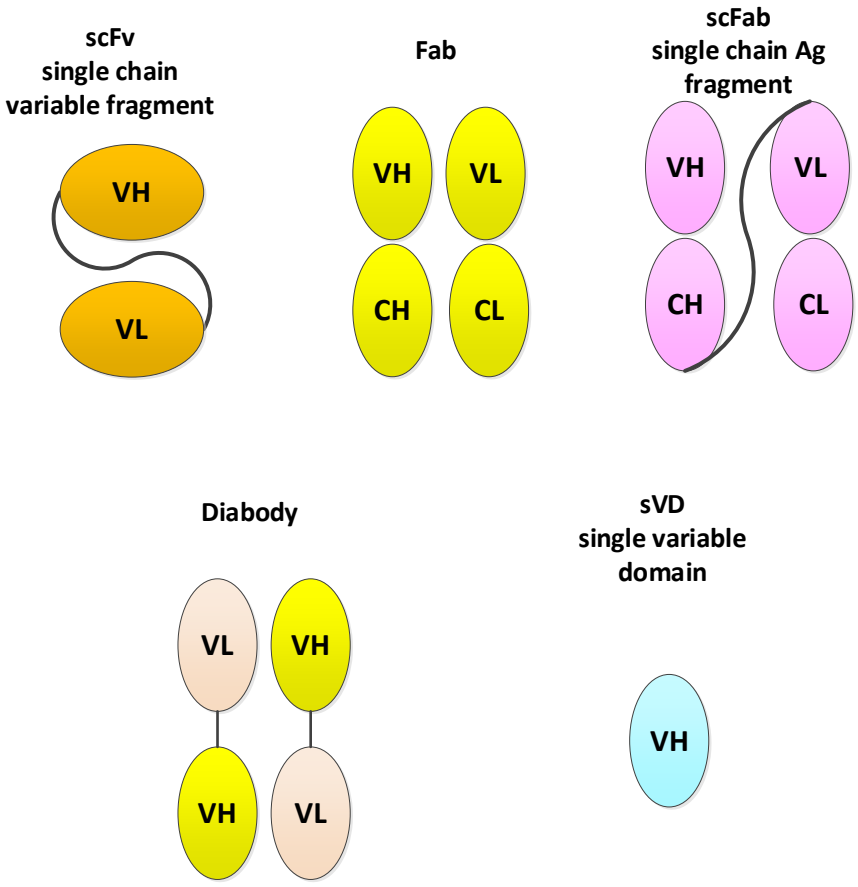
4.1 General Constructs

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 possible 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.

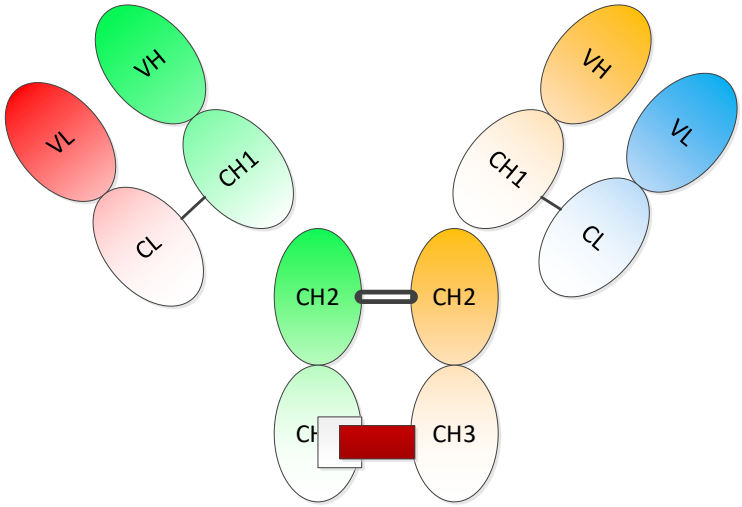
[6] See http://www.hcdm.org/index.php/molecule-information for lists of CD molecules.



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.

4.2 VARIOUS IMPLEMENTATIONS

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

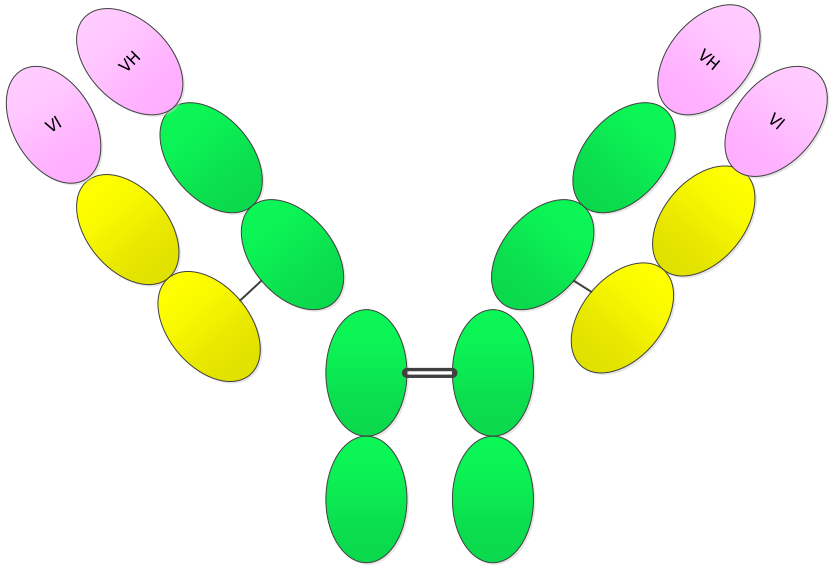
We now examine these in some shape detail.

4.2.1 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.

4.2.1.1 Dual Variable Domains Ig (DVD-Ig)

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.

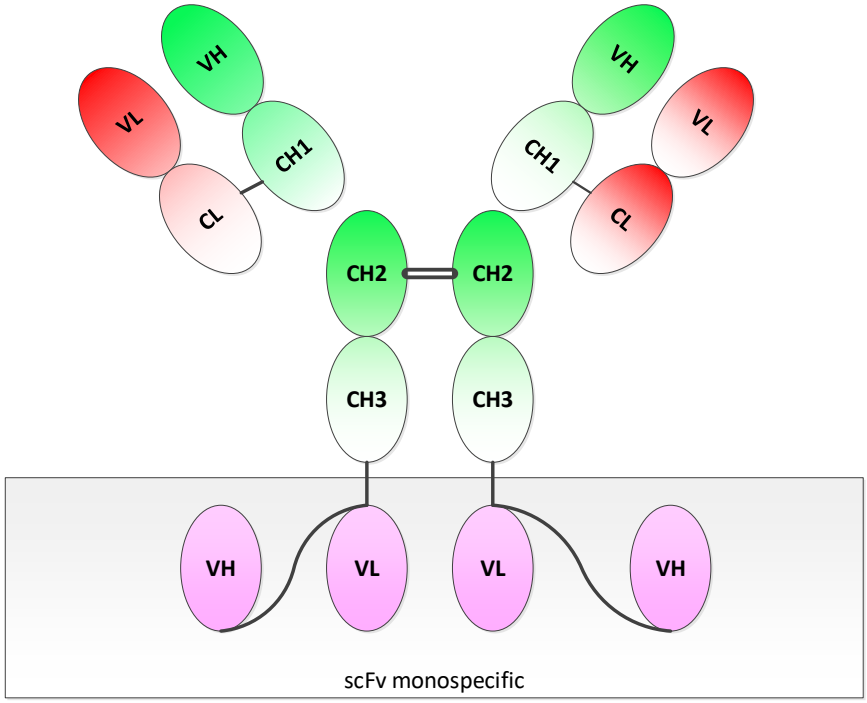


The above has been used in the case of binding VEGF and DLL4 ligands to inhibit angiogenesis in tumor cells[7].

4.2.1.2 scFv-Ig Fusions

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

[7] 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(34): 3861-3870

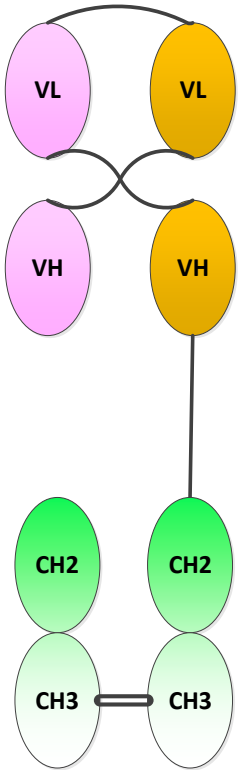


Currently this has been developed to target HER1 and cMET[8].

4.2.1.3 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.

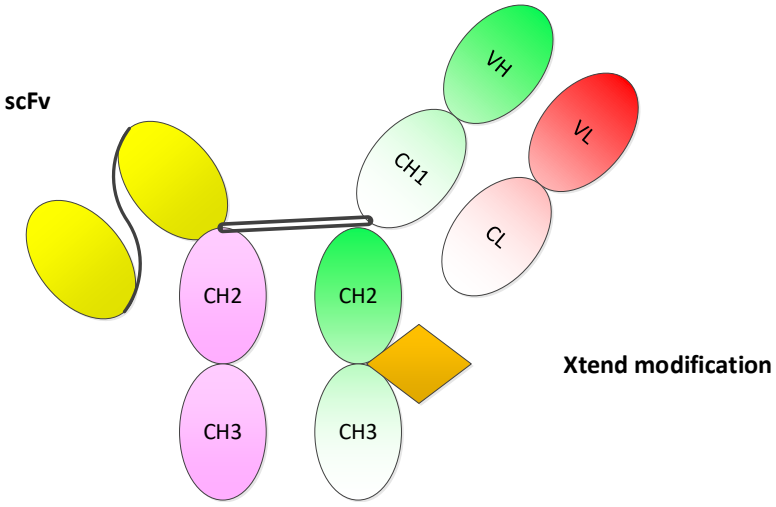
[8] HER1 is also known as EGFR. The protein encoded by this gene is a transmembrane glycoprotein that is a member of the protein kinase superfamily. This protein is a receptor for members of the epidermal growth factor family. EGFR is a cell surface protein that binds to epidermal growth factor, thus 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 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)



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.

4.2.1.4 XmAb

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.



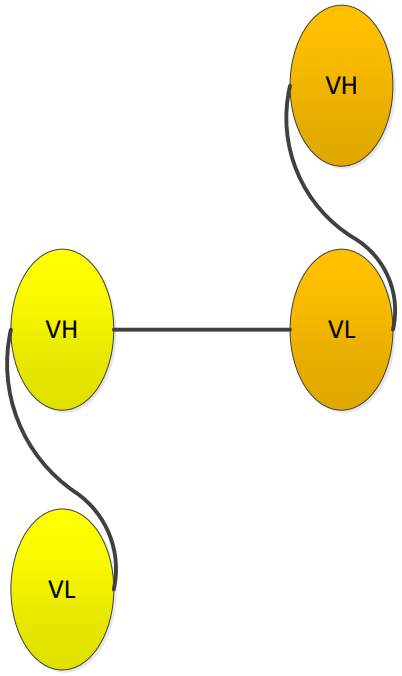
This has a Fab target of LAG-3 and a scFv target of CTLA-4[9].

4.2.2 Fragment Based, Fab

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

4.2.2.1 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:

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

[9] 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)

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.

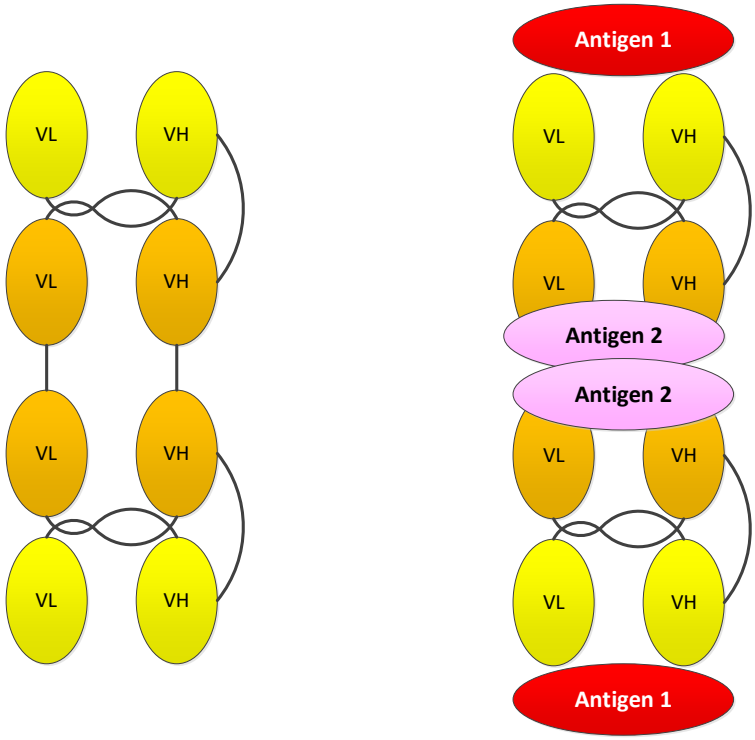
Furthermore, Zahavi and Weiner have recently noted:

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

4.2.2.2 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.



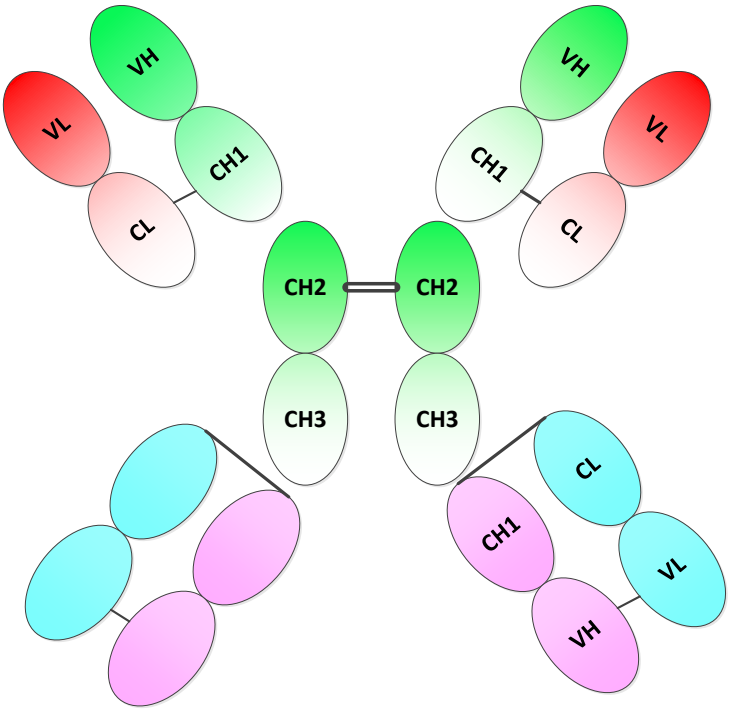
The TandAb form has been developed to block CD3 and CD19.

4.2.3 PreClinical

We now present a mix of preclinical polyAb.

4.2.3.1 biAbFabL

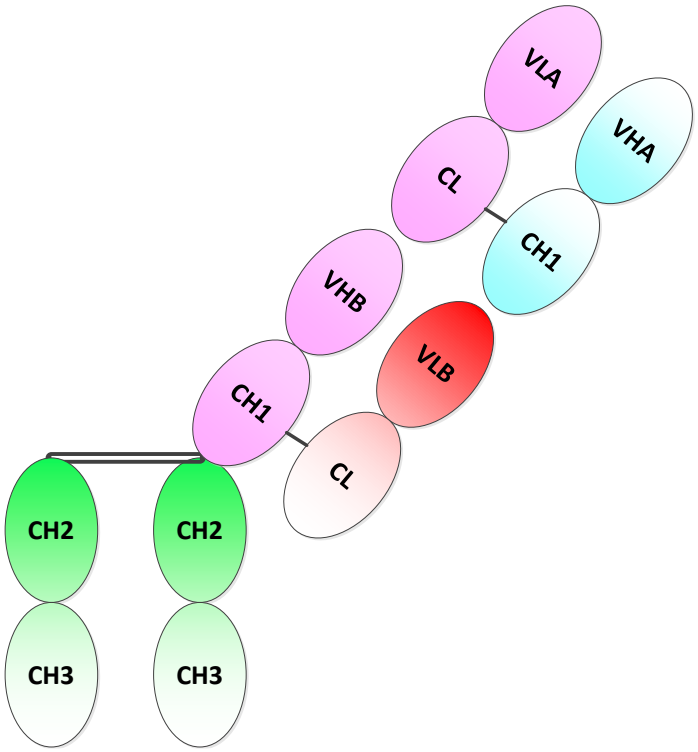
The biAbFabL is shown below and is composed of two Fab domains with a central C domain. Thus the Ab is tetravalent.



The targets for some current developments have been IL-17 and IL-23 inhibition. These target a multiple set of inflammatory disease such as IBD, Chron's, MS and psoriasis.

4.2.3.2 MAT-Fab

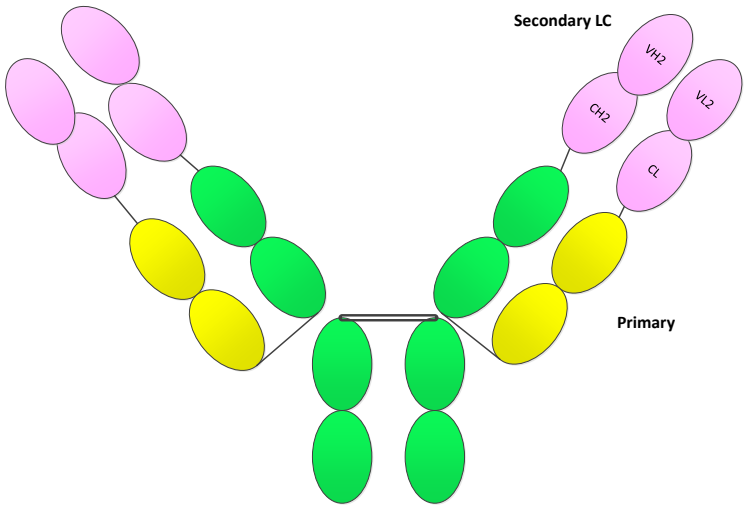
MAT-Fab is a complex tetrameric protein having four protein sections as shown below.



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.

4.2.3.3 Tandem Forms

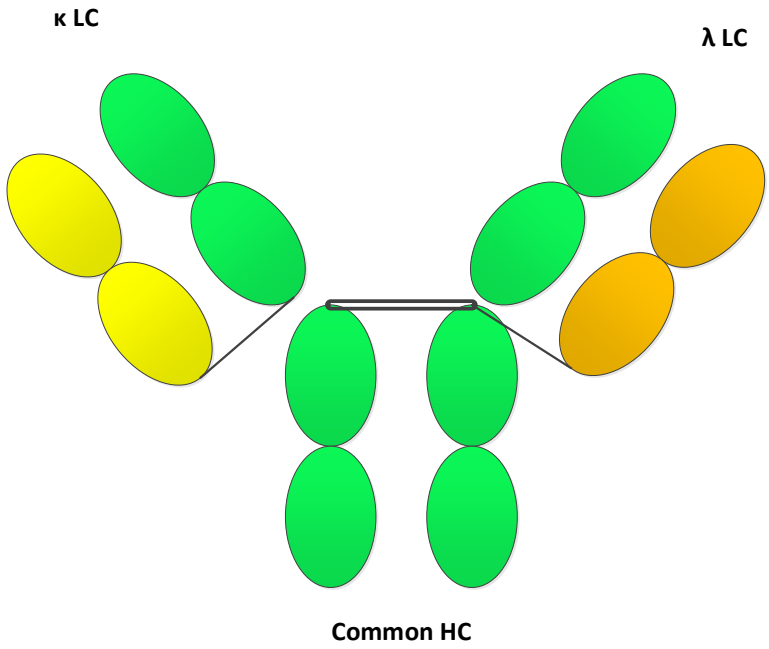
The Tandem form is a “Y-shaped” bispecific antibody format. It closely resembles that of standardized IgG antibodies, and, while being equipped with an Fc region and Fab regions, distinguished itself by having two sets of two Fab regions of different specificity linked in tandem in the Figure below. 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.

4.2.3.4 κλ antibody

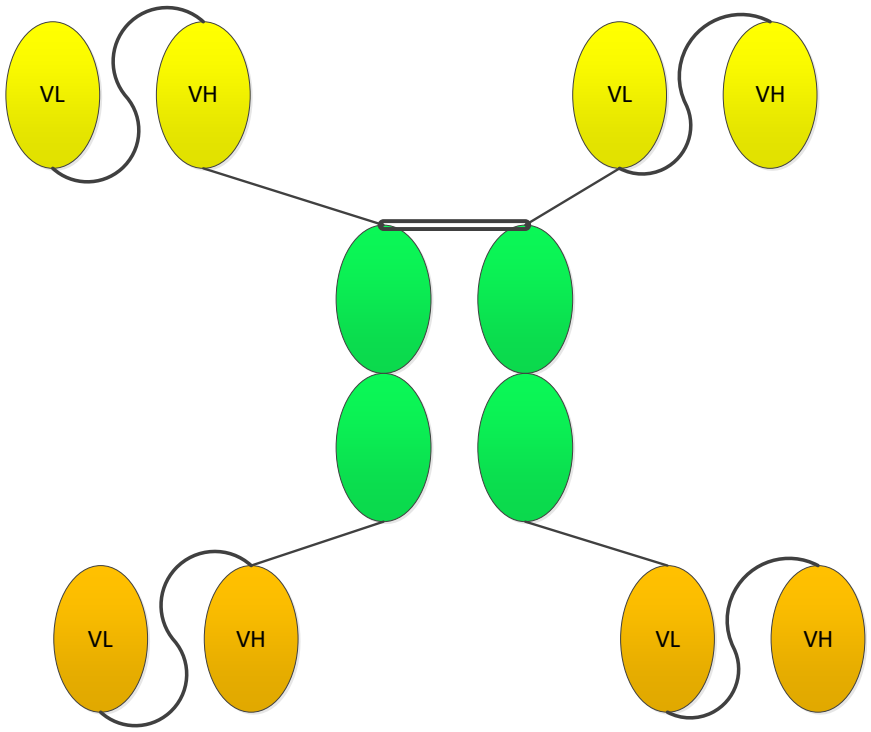
The antibody in this configuration is IgG like in structure except that it has two distinct Fab regions. These two light chains give bi-specific capability.



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

4.2.3.5 ADAPTIR

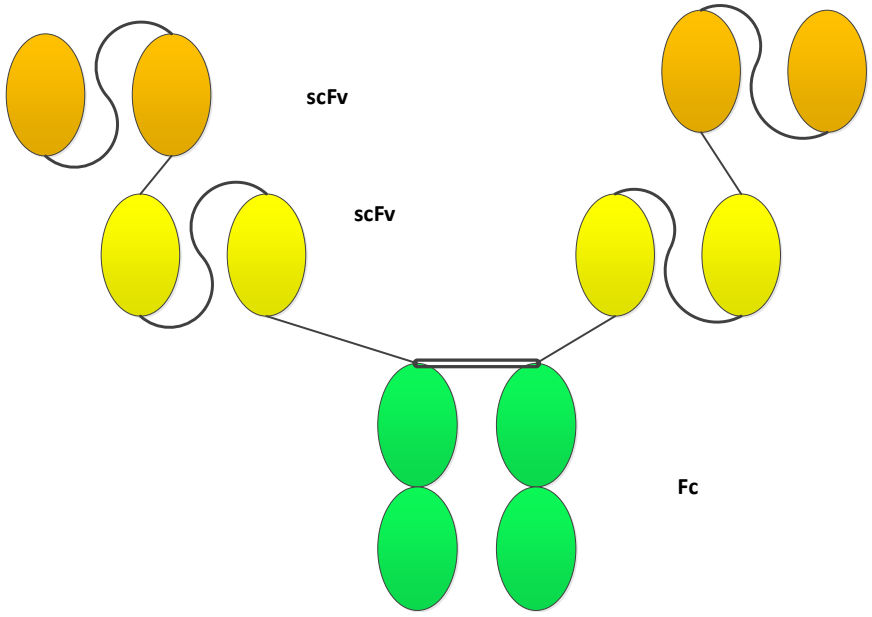
ADAPTIR as a bispecific antibody is comprised of an Fc region and four binding domains with two different specificities. The four binding domains are scFvs and attached in pairs at the amino and carboxyl ends of the Fc region. Thus, the Fc region has two binding domains at each end for binding two different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

4.2.3.6 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.

4.3 PRODUCTION

The challenge is always going from the bench to market. Namely the issue is production, scale and ultimately costs. We have all too frequently see academics with a test tube worth of a therapeutic with no clue as to how or even if this can be scaled up and produced. In our opinion this issue is a critical as the efficacy of the therapeutic. From Husain and Ellerman:

1. Development: *Generation of therapeutic antibodies with a reasonable cost requires that the engineered proteins express at high levels. This is perhaps the earliest critical attribute that novel platforms need to fulfill in order to be viable. Reduction of the cost of goods is a consideration that is also driving the adoption of IgG-like formats compatible with expression in a single cell (some of these are discussed later in the text) or the exploration of processes that allow expression in a single fermentor.*

High level expression is a key factor. In fact it is the first most critical factor in any scalable production format. The second as they not below is purification. As noted the process may produce a great amount of secondary product which is both useless and possibly toxic. They continue:

2. Purification: *Another important aspect is the purification strategy, as a platform that requires a very complex purification scheme would be disadvantageous over other alternatives with a simpler process. Associated with purification, the proper characterization of the desired product and potential contaminant species is important to guarantee the robustness of the production process and the quality of the final product. Depending on the format, the potential formation of*

different unwanted species could be more difficult to detect and quantify, presenting a bigger challenge for analytical groups.

For example, single-cell expression of a bispecific IgG with two different heavy chains and two different light chains, although it simplifies downstream process and reduces costs, may lead to the formation of contaminants with very similar biochemical properties to the intended product and may require advanced methods for their detection .

The contaminants are critical in terms of elimination.

3. Stability: *The chemical and physical stability of the protein is also important, as poor stability may compromise the activity as well as increase the risk of immunogenicity. Physical stability has been a limitation, for example, for the development of some single-chain variable fragment (scFv)- containing bispecific antibody formats due to their intrinsic propensity to aggregate . Several approaches have been developed to overcome this limitation, such as introducing a stabilizing disulfide bond, grafting the complementarity determining regions (CDRs) onto a stable framework, CDR engineering , or by swapping kappa and lambda framework regions*

Once we have delivered the Ab as required we have to be certain it has the desirable pharmacokinetics. Namely it does what it is supposed to and not anything else.

4. Pharmacokinetics: *Most applications of bispecific antibodies require a long half-life in circulation to support sustained drug exposure compatible with infrequent dosing. In some instances, however, a short half-life may provide a safety advantage because in the event of a drug-induced adverse event, the therapeutic can be quickly eliminated from circulation. For example, blinatumomab is a bispecific T cell engager (BiTE) associated with neurological adverse events that are reversible upon discontinuation of dosing. The fast clearance of the format is a benefit in this context .*

Pre-targeting strategies for imaging and radiotherapy also benefit from a short half-life of the targeting antibody. In these applications a bispecific antibody capable of binding the radionuclide and a tumor-assocated antigen is given to patients first. Once the antibody has cleared from circulation, leading to a high tumor-to-blood and tissues ratio, a peptide loaded with the radionuclide is administered. Antibody formats with short half-lives lead to high tumor-to-blood concentration ratios faster than antibody formats with longer half-lives and thus have an advantage in this application.

Targeting the right cells, both the one to be attacked as well as the attacker is critical. As regards to this the authors note:

5. Effector Function: The Fc region of an IgG mediates different cytotoxic mechanisms, such as activation of the classical pathway of the complement through interacting with C1q , as well as activation of cytotoxicity by natural killer (NK) cells and phagocytosis by macrophages through interaction with different Fcγ receptors on the cell surface. Retaining effector functions could be required for some applications; for example, antibodies against infectious agents mediating an increase in pathogen uptake .

On the contrary, interactions of the Fc with immune cells or the complement may lead to undesired toxic effects in other cases, as discussed later in the section on bispecific antibodies for crossing the blood–brain barrier (BBB). *A remarkable advance in the ability to eliminate or modulate these interactions allows for a tailored design of the effector function that best serves the intended application. For example, mutations have been identified that promote the hexamerization of IgGs, leading to a more efficient recruitment of C1q. Also, mutations that increase binding to C1q or FcγRIIIa leading to enhanced complement-dependent cytotoxicity and ADCC, respectively, have been described.*

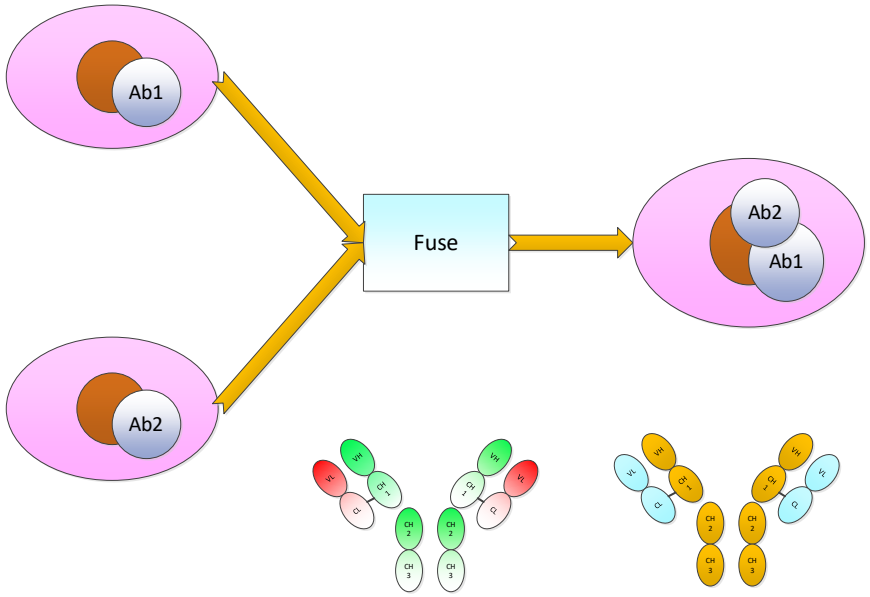
Immunogenicity reflects the patient's own immune response not just favorably but unfavorably. The patient may suffer a cytokine storm effect as one of many responses. In addition may auto immune response are possible as well. They then continue regarding this element:

6. Immunogenicity: *Administration of therapeutic antibodies in humans may trigger the production of* ***anti-drug antibodies (ADAs)****, which may have unwanted consequences. ADAs may reduce or abrogate the activity of the antibodies by blocking their function or by removing them from circulation, or they can lead to toxic responses. The factors involved in determining immunogenicity of a given therapeutic are diverse and complex , but it is a well-established immunological observation that the lower the homology a protein has to the endogenous counterpart, the higher the immunogenic potential, as the chances of containing a T-cell epitope increase. Therefore, when developing or selecting a bispecific antibody format, it is preferable to minimize the differences with a natural IgG. The potential risks of increased immunogenicity associated with highly engineered bispecific formats and some mitigation efforts through the development of reliable methods for the early assessment of immunogenicity are discussed in a separate section below.*

We summarize these production issues as follows:

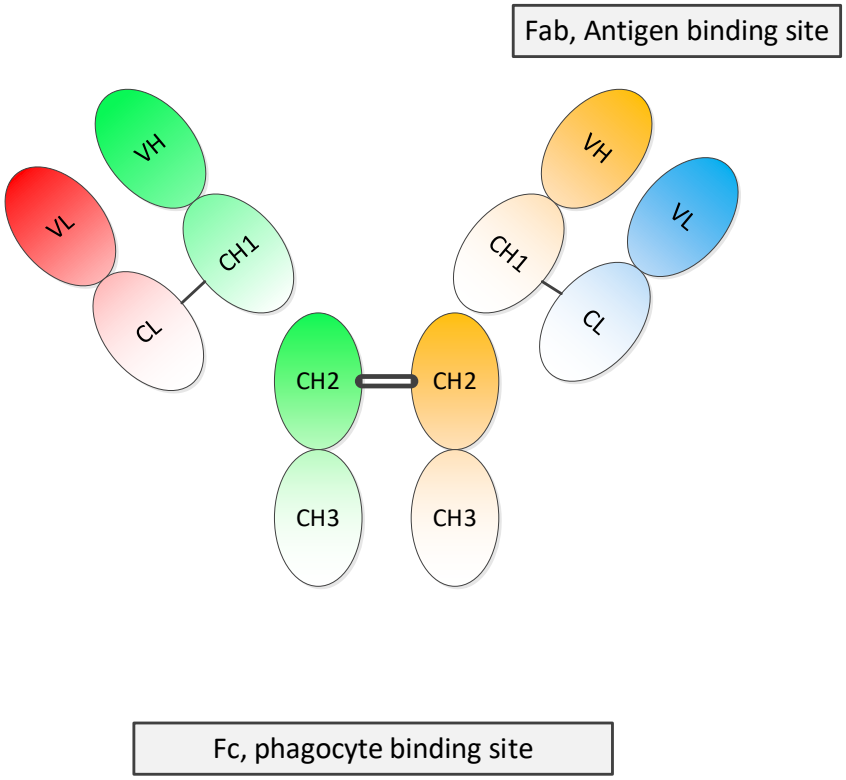
Development	•This is the process whereby we produce the product
Purification	•This is the purification and separation of unwanted elements of the production process
Stability	•This is the taking of the prufied result and placing it in some stable medium for stroage and transport
Pharmokinetics	•This is the assurace via process and adjuvants that the Ab is effectively absorbed and processed
Effector	•This factor is the assuance that the result is properly effective on the target
Immunogenicity	•This is the assurance that secondary immune system factors do not result in morbidity or mortality

The generation of a bi-specific using what is called the Quadroma method is depicted below. We start with two different hybridomas and then fuse them. Each hybridoma has its own IgG and the resultant fused quadroma has the ability to use segments from each IgG and fuse them separately. We depict this approach below:

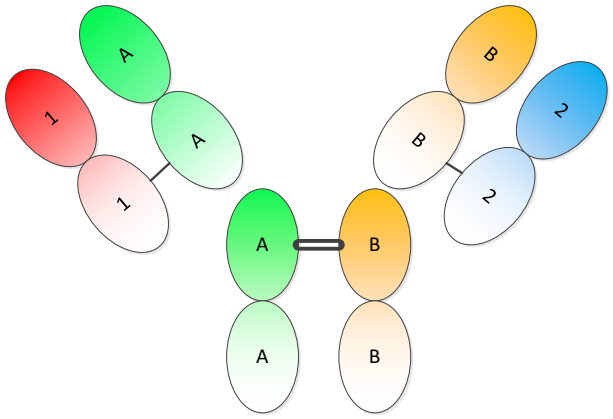


Note that we can used any combination of the Abs produced. We then result in the following. The classic bi-specific may look like the one below. Here we lose the symmetry we see above and contain an Ab with an Fc region, perhaps two, and clearly two separate Ab regions. The two

Ab regions are the basis of the bi-specific power. This if we design it properly we will get two Ab binding sites potentially increasing effectiveness, if we have targeted the correct Ag.



A quick look may give us an understanding of the challenge. Simply, we may have targeted the current Ag on that Fab site and in fact we may have targeted the right pair of Ab. But in producing the bi-specific we may have to use classic symmetric IgG, split them, then allow for the recombination of the separate segments into resultant IgG for our bispecific. The result may be as below, where we have 10 possible results, we want just one, we must separate the one and discard the remaining 9.



1	A	B	1
1	A	B	2
2	A	B	1
2	A	B	2
1	A	A	1
1	A	A	2
2	A	A	1
2	A	A	2
1	B	B	1
1	B	B	2
2	B	B	1
2	B	B	2

There are 12 possible combinations but three are identical so there are but 10. Only one paid consists of all 4 and the remaining 9 are to be excluded.

Wang et al discuss the quadroma in some detail:

Initially, a bispecific antibody was generated by the somatic fusion of two hybridomas... Each hybridoma cell expresses a unique monoclonal antibody with predefined specificity. Then, the two antibody-expressing cells are fused and the resulting hybrid-hybridoma cell expresses the immunoglobulin heavy and light chains from both parents, where assembly allows the formation of both parental and hybrid immunoglobulins. The quadroma technology represents the foundation of bispecific antibody production, but also suffers from low production yields and high product heterogeneity. .

The random assembly of two different heavy and two different light chains can theoretically result in 10 different molecular configurations and only one of those is functional bispecific antibody. The real percentage of functional bispecific antibody by a quadroma cell line is unpredictable and a laborious process is required to isolate the bispecific antibody from the side products. Later, a chimeric quadroma technology was developed by fusing a murine and a rat hybridoma cell line.

The content of chimeric mouse/rat bsAb was significantly enriched due to preferential species-restricted heavy/light-chain pairing in contrast to the random pairing in conventional mouse/mouse or rat/rat quadromas. Furthermore, rat heavy chains did not bind to protein A for purification, while the mouse heavy chains in bsAbs can be eluted at pH 5.8 while the full-size parental mouse Ab can be eluted at pH 3.5 . This feature provided an easy and simple

purification process through protein A and ion-exchange chromatography to isolate the desired bispecific component. With the improvements of quadroma technology, Catumaxomab (anti-EpCAM x anti-CD3) was the first approved IgG-like bispecific antibody in Europe in 2009 for the intraperitoneal treatment of patients with malignant ascites .

Catumaxomab is generated via quadroma technology and composed of mouse IgG2a and rat IgG2b. As a trifunctional antibody, one Fab antigen-binding site binds T-cells via CD3 receptor, the other site binds tumor cells via the tumor antigen epithelial cell adhesion molecule (EpCAM) and the Fc region provides a third binding site to recruit and activate immune effector cells via binding to FcγRI, IIa and III receptors . Nevertheless, Catumaxomab cannot bind to the inhibitory Fcγ IIb receptor. Immunogenicity is another concern—human anti-mouse or anti-rat antibody response are sometimes observed in patients with catumaxomab treatment

4.4 Comparisons and Analyses

The following Table is a list of some bi-specifics and their pros and cons[10]. We list the structure the presence of Fc region, the purification issue and then the advantages and disadvantages.

Name	*Fc region*	*Possible purification strategies*	*Advantages*	*Disadvantages*
DVD-Ig	Yes	PAC/PGC	Avoids HC/LC mispairing	Lower binding affinities
scFv fusions	Yes	Standard processes, depending on conjugated protein	Longer half-life, avoids LC miss paring	Low stability because of linkers
BEAT	Yes	PAC/PGC	No HC/LC mispairing, avoids effects that might cause immunogenicity	Disallows the use of VH3 variable domains without further engineering steps
XmAb	Yes	PAC, IEC	Extended half-life, purification advantages	Possible risk of immunogenicity
BiTE	No	His-tag, PLC	High specificity	Short half-life
TandAb	No	His-tag, PLC	High specificity, improved half-life over BiTE	Short half-life

The following Table depicts some of the types and their targets[11].

[10] See Table 2 Andersson

[11] See Table 3 Andersson et al

Name	Purification strategy	Cell line	Target	Therapy
biAbFabL	PAC + SEC	HEK	IL-17A/F + IL-23	Autoimmune diseases
taFab	PAC + SEC	HEK	IL-17A/F + IL-23	Autoimmune diseases
VCVFc	PAC+SEC	HEK	IL-17A/F + IL-23	Autoimmune diseases
VCDFc	PAC + SEC	HEK	IL-17A/F + IL-23	Autoimmune diseases
MAT-Fab	PAC	HEK	CD3/CD20	Oncology
iBiBody	PAC or PGC	HEK	CD3/FLT3	Oncology (AML)
Tandem forms	PAC or PGC	CHO/HEK	TLR4/TLR2	Unspecified
κλ antibody	PAC + ligand for κ/λ LC	CHO	CD47/mesothelin or CD47/CD19	Oncology
ADAPTIR	PAC + SEC	CHO/HEK	4-1BB/5T4	Oncology
BiIA-SG	PGC	CHO/HEK	CD4/HIV-1 gp160	HIV-1

5 TRI-SPECIFICS

Tri-specific antibodies is the next step in Ab enhancements. For example, if two are good are three better? In a paper by Garfall and June they note:

Antibodies with specificity for one target — called monoclonal antibodies — were the first cancer immunotherapy to achieve widespread clinical use. The therapeutic potency of antibodies can be amplified by engineering them to recognize two distinct molecular targets (termed antigens). These bispecific antibodies can simultaneously bind to cancer cells and immune cells called T cells, and this dual binding directs the T cell to unleash its cell-killing power towards the cancer cell.

Writing in Nature Cancer, Wu et al now report the development of a trispecific antibody, one that has three targets: a cancer cell, a receptor that activates T cells, and a T-cell protein that promotes long-lasting T-cell activity against the cancer cell…. the development of a human antibody that is engineered to bring an immune cell called a T cell into close proximity with a type of cancer cell called a myeloma cell and to boost the T cell's anticancer response.

This trispecific antibody binds three targets:

(i) the protein CD38 on a myeloma cell, and

(ii) the protein CD28 and the

(iii) protein complex CD3 on a T cell.

CD3 is part of the T-cell receptor (TCR), which recognizes abnormal cells by binding molecules called antigens. The binding of CD3 by the antibody drives T-cell activation (without requiring antigen recognition by the TCR), which leads to the killing of the myeloma cell and the production and release of toxic cytokine molecules.

Binding of CD28 by the antibody drives expression of the protein Bcl-xL. Bcl-xL blocks T-cell death, which might otherwise occur if there was prolonged TCR activation in the absence of CD28 stimulation by the antibody.

This is a three way binding, a bi-specific plus one. As Guo et al have noted:

Oncolytic viruses (OVs) are potent anti-cancer biologics with a bright future, having substantial evidence of efficacy in patients with cancer. Bi- and tri-specific antibodies targeting tumor antigens and capable of activating T cell receptor signaling have also shown great promise in cancer immunotherapy. In a cutting-edge strategy, investigators have incorporated the two independent anti-cancer modalities, transforming them into bi- or tri-specific T cell engager (BiTE or TriTE)-armed OVs for targeted immunotherapy. Since 2014, multiple research teams have studied this combinatorial strategy, and it showed substantial efficacy in various tumor

models. Here, we first provide a brief overview of the current status of oncolytic virotherapy and the use of multi-specific antibodies for cancer immunotherapy.

We then summarize progress on BiTE and TriTE antibodies as a novel class of cancer therapeutics in preclinical and clinical studies, followed by a discussion of BiTE- or TriTE-armed OVs for cancer therapy in translational models. In addition, T cell receptor mimics (TCRm) have been developed into BiTEs and are expected to greatly expand the application of BiTEs and BiTE-armed OVs for the effective targeting of intracellular tumor antigens. Future applications of such innovative combination strategies are emerging as precision cancer immunotherapies.

Thus tri-specifics add an additional dimension to the targeting. This may increase specificity and reduce an adverse reactions, yet that is yet to be fully understood. What is clear is that the more we know about a specific cancer molecule the more we can target it and the less damage we may incur in the process.

5.1 FORMATS

As Runcie et al note:

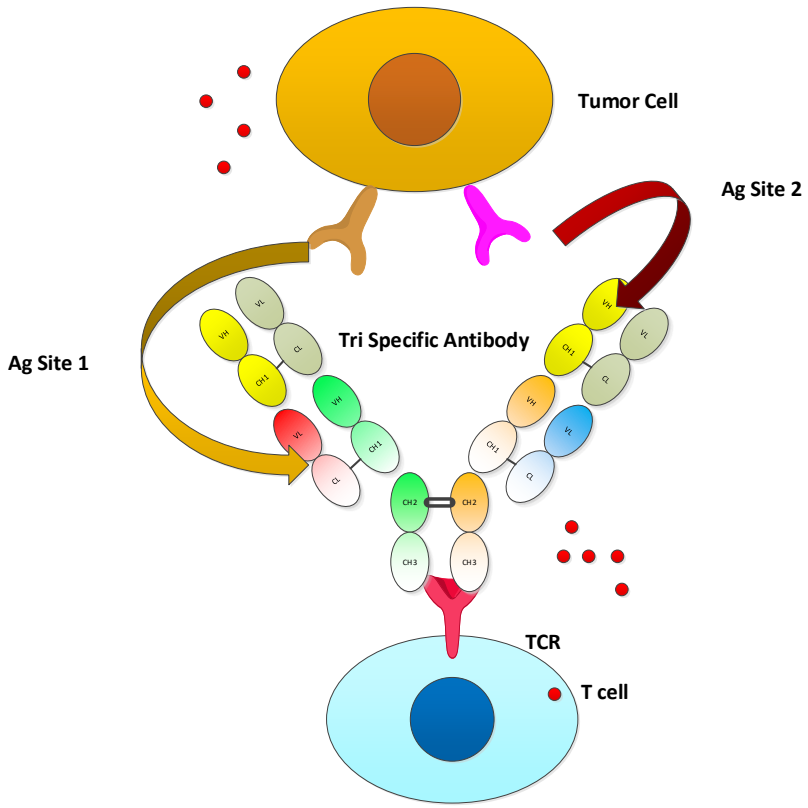
Innovative techniques to harness natural killer cell in immunotherapy have introduced the concept of ***bi-specific killer cell engagers (BiKEs) and tri-specific killer cell engagers (TriKEs). BiKEs are created by the fusion of a single chain variable fragment (Fv) against CD 16 (antigen on natural killer cells) and a single-chain Fv against a tumor associated antigen.***

TriKEs are a combination of a single-chain Fv against CD16 and two tumor associated antigens*.*

These molecules directly trigger NK cell activation through CD 16 amplifying NK cell cytolytic activity and cytokine production against various tumor cell antigen targets. These drugs are currently being investigated in preclinical studies and safety remains a concern with the potential to trigger cytokine cascades …

Even though most polyspecific antibodies have two binding sites (bispecific), there are many new molecules with three or four binding sites. For example, Castoldi et al., have recently developed a tetravalent Fc containing antibody (tetramab) directed against HER1, HER3, c-MET and IGF1R with enhanced antitumor effects in a preclinical model

Runcie et al depict a typical tri-specific as below:



5.2 PRODUCTION

As we did with both MAbs and bi-specific Abs we have the same issue with all polyAbs. Namely that of production. As Brinkmann and Kontermann have noted:

During the past two decades we have seen a phenomenal evolution of bispecific antibodies for therapeutic applications. The 'zoo' of bispecific antibodies is populated by many different species, comprising around 100 different formats, including small molecules composed solely of the antigenbinding sites of two antibodies, molecules with an IgG structure, and large complex molecules composed of different antigen-binding moieties often combined with dimerization modules.

The application of sophisticated molecular design and genetic engineering has solved many of the technical problems associated with the formation of bispecific antibodies such as stability, solubility and other parameters that confer drug properties. These parameters may be summarized under the term 'developability'. In addition, different 'target product profiles', i.e., desired features of the bispecific antibody to be generated, mandates the need for access to a diverse panel of formats.

These may vary in size, arrangement, valencies, flexibility and geometry of their binding modules, as well as in their distribution and pharmacokinetic properties. There is not 'one best format' for generating bispecific antibodies, and no single format is suitable for all, or even most

of, the desired applications. Instead, the bispecific formats collectively serve as a valuable source of diversity that can be applied to the development of therapeutics for various indications. Here, a comprehensive overview of the different bispecific antibody formats is provided. …

Despite the importance of format diversity as a prerequisite for the application of bispecific antibodies for different functions, we want to stress that, as in real life, not all members of a zoo can be easily handled. Some look nice, but are really poorly behaved. For pharmaceutical development, molecules and formats need to be produced in large amounts in a reproducible manner, preferably at high yields with processes that are established or similar to such. The more complex composition does frequently require more extensive optimization of expression systems.

It has to be particularly noted that (in contrast to 'simple' molecules), stability of the expression system and yield are not the only factors to be addressed. In fact, composition of the bispecific antibodies and presence or absence of undesired side products can be of equal (or higher) importance. In addition to being fit for production and upstream/downstream processing, bispecific antibodies need to be well defined, stable and overall 'well behaved' to become drugs.

Many of these parameters are addressed under the term 'developability'. Bispecific antibodies that fulfill developability criteria would be stable (e.g., against thermal denaturation) with low tendency to aggregate, low tendency to accumulate chemical deviations and (dependent on the mode of application) preferentially able to be formulated at high concentrations without viscosity issues.

Thus, designing, optimizing and characterizing bispecific antibodies with desired specificity and functionality is just the beginning of a long process. Converting such molecules into drugs is a difficult endeavor. That part, however, is the key without which bispecific antibodies would remain only exotic members of a zoo, and not drugs.

The above authors present a somewhat complete taxonomy of bi-specifics. The actual targeting of them is a more complex task especially in the matter of production. Chen et al further note regarding production:

With the current biotherapeutic market dominated by antibody molecules, bispecific antibodies represent a key component of the next-generation of antibody therapy. Bispecific antibodies can target two different antigens at the same time, such as simultaneously binding tumor cell receptors and recruiting cytotoxic immune cells. Structural diversity has been fast-growing in the bispecific antibody field, creating a plethora of novel bispecific antibody scaffolds, which provide great functional variety. Two common formats of bispecific antibodies on the market are the single-chain variable fragment (scFv)-based (no Fc fragment) antibody and the full-length IgG-like asymmetric antibody.

Unlike the conventional monoclonal antibodies, great production challenges with respect to the quantity, quality, and stability of bispecific antibodies have hampered their wider clinical application and acceptance. In this review, we focus on these two major bispecific types and

describe recent advances in the design, production, and quality of these molecules, which will enable this important class of biologics to reach their therapeutic potential. …

This review has focused on the design, production, and quality of bispecific antibodies. A key challenge is how to produce uniform bispecific antibody with high quality and limited or negligible side products and impurities. For scFv-type bispecifics, the protein stability and tissue penetration ability vary and depend on different types of scFv antibody. Furthermore, with multiple host options to choose from, the determination of the most suitable system depends on the specific scFv antibody size, amino acid sequence, protein conformation, solubility, stability, purification, and scalability. For IgG-like full-size bispecific antibody, the production of pure heterodimer is achieved by complete heavy chain and light-chain heterodimerizations.

Knobs-into holes method is an efficient means with which to associate different heavy chains. The common light chain and CrossMab technology are also useful approaches for varying light chain and heavy-chain assembly. More recently, co-culture and cell-free systems are also emerging as complementary production platforms to generate bispecific antibodies readily. Advanced protein and production engineering technologies in the antibody field have boosted the development of bispecific antibodies and their derivatives, which represent one of the fastest-growing next-generation of antibody therapeutics . Diversity has been obtained in the bispecific antibody structure design both in the scFv- and IgG-like formats or by using a combination of both.

Furthermore, the addition of small molecules such as aptamers, affibodies, and synthetic drugs can further expand their applicability, creating a plethora of novel bispecific antibody-related products. Bispecific antibodies have found wide applicability to immunotherapy for cancer treatment, and these diverse molecules have the potential to treat other diseases, such as infections, acquired immune deficiency syndrome (AIDS) and genetic diseases as well as serving for medical diagnosis purposes. Looking forward, with continuous efforts to improve their design, production, and purification on an industrial scale, bispecific antibodies will represent an increasing share of the therapeutics in the market with the capacity to reach their full potential as a complementary approach to the conventional therapy in the next decade

As Wang et al note:

Appropriate host platforms are determinant to the efficient expression and production of scFv antibodies, and there exist several different viable platforms for scFv expression including bacteria, yeast, mammalian cells, insect cells, plant and cell-free systems. Given that bispecific scFvs are composed of two or more scFv molecules, the various expression hosts for the bispecific scFvs may vary from those used for the production of scFv single molecules.

The "best" expression system for bispecific scFv proteins is yet to be determined because differences in size, amino acid sequence, and conformation of the recombinant protein make it difficult to conclude a universal expression system that optimizes the yield and quality of the protein, which can be affected by many factors such as solubility and stability However, several studies listed in Table 1 have reported successful expression of bispecific scFv and its fusion molecules using bacterial and mammalian systems. E. coli is one of the most widely used hosts

for scFv expression. Some of the major advantages of using E. coli include its rapid growth, cost efficiency, high heterologous protein productivity, well-understood genetics as well as easy genetic manipulation.

Unlike the glycosylated whole antibody protein, scFv molecules are much easier to produce in bacteria. However, challenges still remain for harnessing this high-yield expression system, one of which is insufficient protein solubility. It was reported by multiple studies that proteins produced from the E. coli expression system result in misfolding and inclusion body. This inefficiency in producing soluble scFv is known to be caused by the lack of chaperon and post-translational machinery and the reducing environment of E. coli cytoplasm which prevents disulfide bonds to be formed, and for scFv molecules, formation of intra-domain disulfide bonds is essential for the key structure known as the "immunoglobulin fold".

Therefore, successful expression of functional scFv molecules from E. coli systems usually requires additional procedures or modifications. For example, subsequent protein refolding and recovery steps can be integrated into the process, including solubilization treatment with agents such as urea and guanidine hydrochloride, and a step to refold solubilized protein by removing solubilization agents by methods such as dialysis. Gruber et al. reported the production of bispecific scFv in E. coli with these refolding steps. The solubility of scFv molecules can also be improved by secreting them into the bacterial periplasm that has an oxidizing environment, through genetically attaching the secretory signal sequence to the N-terminus of scFv sequence. A number of studies have reported the periplasmic expression of BiTE type molecules in E. coli

Wang et al continue:

Heavy Chain and Light-Chain Assembly While deliberate modifications of Fc CH3 domains enable correct heavy-chain heterodimerization, using two different light chains still results in a low yield of desired bispecific antibodies (the generation of four different combinations, with only one being bispecific).

Advanced approaches have, therefore, been developed to allow the correct pairing of light chain and heavy chain to resolve the improper heavy chain and light-chain interaction problem, such as the common light-chain method and CrossMab to swap the VH and VL Fab fragments partially. First, a common light-chain strategy was applied to assemble IgG-like bispecific antibodies which can be combined together with the knobs-into-holes approach.

The mechanism of a common light-chain strategy is based on the fact that antibodies discovered from phase display screening against diverse antigens often share the same VL domain, reflecting the very limited size of the L chain repertoire in the phage library. One of the great advantages of the common light-chain format is that it allows the use of methods that simplify the antibody engineering and the purification process in industrial production . For example, based on computational prediction, one Fc variant pair dubbed "DEKK" consisted of substitutions L351D and L368E in one heavy-chain combined with L351K and T366K in the other drove efficient heterodimerization of the antibody heavy chains . Additionally, using a common light chain, the bispecific antibody MCLA-128, targeting human EGF receptors 2 and 3, was

produced and purified with a standard CHO cell culture platform and a routine purification protocol under Good Manufacturing Practice (GMP) conditions .

More recently, a full-length bispecific IgG-like bsAb was approved in 2017 was emicizumab (Hemlibrafi) for the treatment of Hemophilia A patients .

Engineered on the structure of humanized IgG4, emicizumab mimics the function of activated FVIII to restore the FVIII binding to factor IX (FIX) and factor X (FX), which is missing in Hemophilia A patients. Large-scale manufacturing of emicizumab was achieved by a combination of three antibody engineering strategies-a common light chain to assemble heavy and light chain, changing the charges of two different heavy chains to facilitate antibody purification, and the application of electrostatic steering of two different heavy chains to promote expression of heavy chains in cells .

Currently, numerous common light chain and common heavy-chain discovery platforms have been developed to enable the effective generation of antibodies for bsAb assembly. These include but are not limited to transgenic mice with a fixed single light chain as well as screening phage display libraries with common heavy chain. Therefore, the application of a common light chain is becoming increasingly popular in this field in order to overcome the stability, yield, and immunogenicity problems of bispecific antibodies. However, this approach may lower flexibility in antibody engineering, which limits antibody optimization in some cases . Furthermore, the screening process for common light chain requires animal immunization and/or phage display, which may be problematic due to time and development costs

5.3 USAGE

We now examine the specific application of tri-specifics. Much of the work here is recent and is subject to change. Guo et al note:

Trispecific antibodies binding to NK or T cells have also been explored to treat cancer (Figure 2). In one study, Vallera and colleagues designed IL-15 trispecific killer engagers (TriKE) based on their previous BiKE construct . This TriKE contains a single-chain scFv against CD16 and CD33 to create an immunologic synapse between NK cells and CD33+ myeloid targets, as well as an IL-15 crosslinker that produces a trispecific engager to induce expansion, priming and survival of NK cells. When compared with the 1633 BiKE, the 161533 TriKE induced superior NK cell cytotoxicity, degranulation, and cytokine production against CD33+ HL-60 promyelocytic leukemia targets. In addition, the TriKE increased NK cell survival and proliferation.

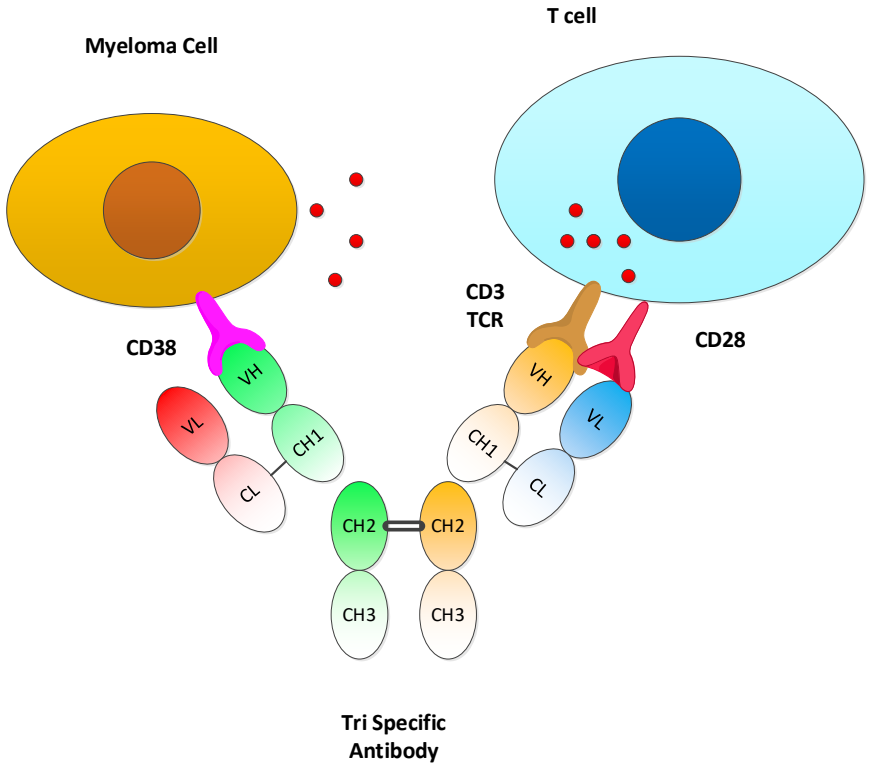
Specificity was demonstrated based on the selective ability of the 1615EpCAM TriKE to kill CD33-EpCAM+ target cells. In vivo, the 161533 TriKE exhibited superior antitumor activity and induced in vivo persistence and survival of human NK cells in an HL-60-luc tumor model for at least 3 weeks. CD28 costimulation provides another opportunity for therapeutic intervention, despite a checkered history in past therapeutic applications . A trispecific antibody against CD3, CD28 and CD38 enhanced both T cell activation and tumor cell targeting .

The engagement of both CD3 and CD28 affords specific T cell activation, limits apoptosis/anergy, while provision of the anti-CD38 Ab recognizes myeloid cells as well as some lymphomas and leukemias. In a humanized mouse model, this trispecific T cell engager antibody (TriTE) treatment suppressed myeloma growth. It also stimulated memory/effector T cell proliferation and reduced Treg cell levels in non-human primates. Collectively, these studies suggest that trispecific antibodies represent a promising platform for cancer immunotherapy. …

The design of a triple specific T cell engager (TriTE) antibody and how it links the T cells to the targeted cancer cells. This trispecific antibody binds three targets: the CD38 protein on a myeloma cell, and the protein CD28 and the CD3 protein complex on a T cell (the antibody's target-binding domains are shown in red, blue and yellow, respectively). CD3 is a component of the T cell receptor (TCR).

The binding of CD3 by the antibody drives T cell activation without requiring antigen recognition by the TCR, which leads to the killing of the myeloma cell and the production and release of toxic cytokine molecules.

The following is a Figure adapted from Guo et al describing this process:



This is further enhanced by the results of Wu et al who note:

Despite the significant therapeutic advances provided by immune-checkpoint blockade and chimeric antigen receptor T cell treatments, many malignancies remain unresponsive to

immunotherapy. Bispecific antibodies targeting tumor antigens and activating T cell receptor signaling have shown some clinical efficacy; however, providing co-stimulatory signals may improve T cell responses against tumors.

Here, we developed a trispecific antibody that interacts with CD38, CD3 and CD28 to enhance both T cell activation and tumor targeting. The engagement of both CD3 and CD28 affords efficient T cell stimulation, whereas the anti-CD38 domain directs T cells to myeloma cells, as well as to certain lymphomas and leukemias. In vivo administration of this antibody suppressed myeloma growth in a humanized mouse model and also stimulated memory/effector T cell proliferation and reduced regulatory T cells in non-human primates at well-tolerated doses.

Collectively, trispecific antibodies represent a promising platform for cancer immunotherapy…. In developing this trispecific antibody for myeloma therapy, we determined that the additional CD28 specificity not only affected T cell survival but also directed T cells more efficiently to myeloma cells. This second target antigen contributed to more effective tumor recognition and lysis, even in myelomas that express lower levels of CD38. Low CD38 expression may arise naturally or could be selected by previous anti-CD38 antibody therapy62. The CD28 specificity in the CD38 trispecific antibody therefore plays a dual role in protecting against tumor growth.

Enhanced killing based on improved recognition of target cells through the recognition of multiple targets illustrates another feature of trispecific antibodies that can increase selectivity and reduce non-specific toxicity. The flexibility of this format may allow for inclusion of other cell-surface antigens that can be applied to other tumor targets—either with other T cell engagers, antibody drug conjugates or natural killer cell engagers. The recognition of independent target sites with multispecific antibodies can be applied to a variety of malignancies and offers a platform to stimulate immunity through mechanisms that promote the recognition of key cell-surface molecules and stimulation of relevant effector responses.

This trispecific thus represents a first-in-class T cell activation molecule that delivers two signals to activate T cells while targeting them specifically to tumor targets. This T cell stimulation and targeting therapeutic is the prototype of a broader platform that can be used to treat different cancers and infectious diseases. Although T cell engagers have been used successfully in humans, their therapeutic efficacy is limited in part because optimal T cell signaling cannot be achieved with previous technologies. This dual activation platform provides a mechanism not only to optimize T cell activation, but also to improve targeting. This multi-specific strategy also stimulates molecular co-signaling not readily achieved by simply combining monoclonal antibodies. The approach could also be applied to other targeted therapies for cancer immunotherapy, including, for example, CAR T cells.

While the CD38 trispecific antibody has not been evaluated in humans yet, the preliminary characterization of this Ab and a previously described HIV therapeutic in NHPs suggests that they behave analogously to conventional antibodies13. While anti-drug responses could potentially arise in vivo, a bispecific antibody to the human cytokines IL-4 and IL-13, using a related format and linkers, indicated that dual variable region antibodies have been safe and well tolerated in humans. The inclusion of multiple specificities into a single protein for cancer immunotherapy simplifies clinical development.

Clinical trials are currently planned to assess the full potential of the trispecific antibody platform, both for HIV and cancer, based on supportive data from NHP studies and the previous human bispecific antibody experience. The in vitro and animal model data presented here suggest that further clinical investigation is warranted. The flexibility of the platform illustrated by this CD38 trispecific antibody will facilitate the rational design of improved therapies for diverse cancers.

6 PRODUCTIONS ISSUES

One of the challenges of Ab development and even more so with poly-specifics is the selection process and the overall production and validation. We briefly discuss some of these issues. We also provide some detail in the Appendix on a multiple set of bi-specifics. The caution is that moving from a one-off Lab environment into a full scale pharmaceutical one is complex and the challenge may set back many good advances. The issue of scale is critical. There is always a potential mismatch between the science side and the engineering side, between the bench and the batch. One of the major challenges in scaling up is maintaining quality control of the process. That is often an inhibiting step.

6.1 TARGETING

Selection and production of targets is a challenge. Listek et al have recently provided an improved and more efficient approach. They note:

The use of monoclonal antibodies is ubiquitous in science and biomedicine but the generation and validation process of antibodies is nevertheless complicated and time-consuming. to address these issues we developed a novel selective technology based on an artificial cell surface construct by which secreted antibodies were connected to the corresponding hybridoma cell when they possess the desired antigen-specificity.

Further the system enables the selection of desired isotypes and the screening for potential cross-reactivities in the same context. for the design of the construct we combined the transmembrane domain of the eGf-receptor with a hemagglutinin epitope and a biotin acceptor peptide and performed a transposon-mediated transfection of myeloma cell lines. the stably transfected myeloma cell line was used for the generation of hybridoma cells and an antigen- and isotype-specific screening method was established. The system has been validated for globular protein antigens as well as for haptens and enables a fast and early stage selection and validation of monoclonal antibodies in one step….

Antibodies are well known as universal binding molecules with a high specificity for their corresponding antigens and have found, therefore, widespread use in very many different areas of biology and medicine.

Most murine antibodies are produced today by means of the hybridoma technique as monoclonal antibodies or with the help of antibody gene libraries and display techniques as recombinant antibody fragments. Both methods have intrinsic advantages but also difficulties such that they are restricted to specialized laboratories and companies. Currently, the reliability of monoclonal antibodies was critically discussed in several publications which is related to a growing demand of better validation and characterization of these molecules Especially the hybridoma technique which results in full-length monoclonal antibodies can be cumbersome, labour-intensive and time-consuming. Although several improvements have been tried in the course of the past years, the basic method is still very similar to the original method. The critical issue in the development of antigen-specific hybridomas is the lack of any direct connection between the hybridoma cell

and the released antibody. Therefore, it is necessary to perform limited dilution techniques in order to separate single cells to ensure monoclonality. Unfortunately, this process could not be combined with a simultaneous, proper validation of the desired antibodies because the concentration in the supernatants are often very low at the early beginning of culture. To facilitate the isolation of specific antibody-producing hybridomas, a method has to be established which temporarily restricts the cells from releasing the antibody into the culture medium and thus retaining the genotype (the antibody-coding genes) and the phenotype (the produced antibodies) in one entity.

Such precondition can easily be fulfilled when recombinant antibody fragments are isolated, e.g. by phage display techniques. To confer this basic principle to the hybridoma technique would require to capture the synthesized antibody on the surface of the synthesizing hybridoma cell. To realize this, a covalent surface labeling of antibody-producing cells with biotin was accomplished in the past, which allowed the isolation of specific cells by means of avidin- or streptavidin-conjugated ligands binding the released antibodies. However, chemical surface labeling is very often unpredictable and may disturb normal functions and the vitality of the cells.

Selection is assisted by The European Antibody Network in the paper by Roncador et al which states:

Antibodies are widely exploited as research/diagnostic tools and therapeutics. Despite providing exciting research opportunities, the multitude of available antibodies also offers a bewildering array of choice. Importantly, not all companies comply with the highest standards, and thus many reagents fail basic validation tests. The responsibility for antibodies being fit for purpose rests, surprisingly, with their user.

This paper condenses the extensive experience of the European Monoclonal Antibody Network to help researchers identify antibodies specific for their target antigen. A stepwise strategy is provided for prioritising antibodies and making informed decisions regarding further essential validation requirements. Web-based antibody validation guides provide practical approaches for testing antibody activity and specificity. We aim to enable researchers with little or no prior experience of antibody characterization to understand how to determine the suitability of their antibody for its intended purpose, enabling both time and cost effective generation of high quality antibody-based data fit for publication

6.2 DESIGN

As Norman et al note:

Antibodies continue to dominate the field of biotherapeutics with an increasing number of new clinical approvals each year. Current approaches to bring these molecules to the market have remained experimentally focused, with animal immunization and surface display technologies accounting for the majority of molecules developed to date. The increasing amount of antibody-specific data in the public domain facilitates the maturation of computational antibody design methods, resulting in a growing uptake as part of standard pharmaceutical discovery processes.

Computational methods are unlikely to replace the entire discovery process. Indeed, their largest added value will continue to be in providing time and cost-efficient ways of guiding experimental methods. Structural modelling can offer insight on exposed residues to be used for mutagenesis to either optimize binding, reduce immunogenicity or provide information on hydrophobicity patches related to detrimental biophysical properties.

Predicting interface information can provide an initial guide for experimental epitope mapping efforts or offer a starting point for a therapeutic campaign by providing the basis for focused surface display libraries to design a novel antibody binder for a given epitope. Exploiting the vast amount of data generated by NGS will facilitate the derivation of more reliable 'humanness' and 'developability' profiles with which to guide antibody therapeutic discovery. Existing computational antibody design knowledge and tools may benefit emerging biotherapeutic modalities akin to antibodies, such as nanobodies.

However, despite the similarity between antibodies and nanobodies, systematic benchmarking will still be needed to determine whether development of nanobodies can benefit from computational antibody protocols in their current form or whether they need to be adjusted accordingly. Holistically, benchmarking of bioinformatic antibody methods on a par with existing protein-generic initiatives such as CASP, CAPRI or CAMEO will benefit the entire computational antibody field. Antibody-specific benchmarking challenges will emphasize the shortcomings and advantages of each method and enable improvements to be developed in a focused manner, specifically with regard to their utility in therapeutic development process.

Further progress in the development of antibody-specific computational tools will be associated with access to more and diverse data in the public domain. It will become increasingly important that these data adhere to information management and reusability best practices. Such efforts are exemplified by AIRR community, which aims to standardize the increasing amount of antibody NGS depositions and their metadata , and from a broader perspective by the adoption of scientific data management principles such as FAIR .

Organizations involved in the discovery and development of antibody therapeutics have a unique opportunity to catalyze the development of the computational antibody methods by participating in data sharing and benchmarking efforts. Publishing proprietary data, which has no or little commercial value, generated in the process of developing a candidate therapeutic may yield a higher return in the form of better computational methods. As the importance of antibodies as therapeutics grows, faster and more accurate computational methods are set to become even more tightly integrated into therapeutic development processes, thus accelerating the delivery of new medicines to patients.

6.3 CELL FUSION

Brinkmann and Kottermann note:

Fusion of a Fab arm to an IgG, i.e., a tandem arrangement of Fab arms fused to a Fc region, was used to generate tetravalent (Fab)2-Fc fusion proteins by expressing an Fd-extended heavy chain together with a light chain (Tandemabs). This format can be applied to generate

tetravalent, bispecific (Fab)2-Fc fusion proteins. Here, production of bispecific molecules also faces the light chain problem because only one of the eight possible combinations is bispecific. Brunker and coworkers generated a bispecific IgG-Fab molecule by applying the CrossMab technology to the second Fab fused through a (G4S)4 connector to the C-terminus of an IgG molecule.

This approach, in which the VH of the fused Fab is linked to a CL domain, and the VL domain to a CH1 domain, was applied to generate a tetravalent, bispecific antibody directed against fibroblast activation protein (FAP), for targeting of activated tumor stroma fibroblasts, and DR5, to induce apoptosis by activation of this death receptor.

Differences were observed for a VHCL and VLCH1 configuration. The VLCH1 format, with the VL domain fused to the C-terminus of the HC, showed, compared to the parental antibody, reduced binding to FAP, while binding activity was retained in the VHCL format (VH domain fused the HC). In combination with a knobsinto-holes Fc region, this approach can also be applied to generate trivalent, bispecific IgG-Fab fusion proteins.243 The orthogonal Fab design described above184 was applied to generate a tetravalent, bispecific Fab-IgG fusion protein Here, the Fd chain of the outer Fab arm is linked to the VH of the heavy chain, i.e., the inner Fab arm using a (G4S)5 linker, and used to produce a bispecific Fab-IgG targeting two epitopes on HER2 (derived from pertuzumab and trastuzumab).

Compared to a scFv-IgG fusion protein of the same specificities, superior biophysical properties and unique biological activities were reported. Fab-IgG fusion proteins were also generated using the charged residue mutations or hydrophobicity-polarity-swap mutations introduced into the CH1-CL interface. In this study, the Fd fragment of a first antibody was fused to a hinge sequence (either wild-type or with the two cysteine mutated to serines), then connected by a STPPTPSPSGG linker to the N-terminus of the heavy chain carrying the CR3 or MUT4 mutations in the CH1 domain.

The cognate light chain of the inner Fab binding site carried the corresponding mutations. Thus, molecules with heavy chains containing the wildtype hinge sequence were covalently linked by disulfide bonds in the additional hinge between the two Fd sequences. Functionality was demonstrated for a tetravalent, bispecific Fab-IgG fusion protein directed against HLA-DR (outer Fab arm) and CD5 (inner Fab arm). Good stability, limited aggregation and in vivo activity was observed for the CD5 x HLA-DR(CR3) molecule.

Molecules with a cysteine-free hinge in the inter-Fab region showed a somewhat reduced binding and where slightly less effective, e.g., in complement activation. As described above, Fabs arm with the Ca and Cb domains from the TCR were applied to link a second Fab arm either to the N-terminus or the C-terminus of the heavy chain through a (G4S)4, as shown for a bispecific antibodies derived from trastuzumab and pertuzumab.203 Additional mutation were introduced into the VL domains (Y36F) to weaken the VH-VL interaction combined with a charge-charge interaction between the VL and VH domains (VL Q38D, VH Q39K) in one of the Fab arms to further facilitate correct pairing of the cognate Fab arms. The DNL method described above was used to generate hexavalent, bispecific IgG-Fab4 molecules, which are produced by mixing DDD2 Fab fusion proteins with a AD2 IgG fusion protein under redox

conditions, followed by purification with protein A. Thus, this approach results in bispecific 2 C 4 molecules, with the two binding sites of the IgG directed against the first antigen and the four binding sites of the Fab arms directed against the second antigen. DDD2 can be fused either to the N-terminus or the C-terminus of the Fab Fc chain.

6.4 CHROMATOGRAPHY

The separation is performed via chromatographic means. Namely the fixed element has the ability to attract only the desired Ab while the others are flushed. Then the resultant is flushed ad repurified.

7 TREATMENTS

There has been a significant amount of work examining what these polyspecific Ab can be used for. We examine some of these herein.

For example, in cancers, a recent paper by Shim noted:

The ability of monoclonal antibodies to specifically bind a target antigen and neutralize or stimulate its activity is the basis for the rapid growth and development of the therapeutic antibody field. In recent years, traditional immunoglobulin antibodies have been further engineered for better efficacy and safety, and technological developments in the field enabled the design and production of engineered antibodies capable of mediating therapeutic functions hitherto unattainable by conventional antibody formats.

Representative of this newer generation of therapeutic antibody formats are bispecific antibodies and antibody–drug conjugates, each with several approved drugs and dozens more in the clinical development phase. In this review, the technological principles and challenges of bispecific antibodies and antibody–drug conjugates are discussed, with emphasis on clinically validated formats but also including recent developments in the fields, many of which are expected to significantly augment the current therapeutic arsenal against cancer and other diseases with unmet medical needs….

The polyAb are quite challenging. Even the simpler bi-specific provide yield challenges and quality control issues. As we had shown with the quadroma, 90% of the yield is eliminated. Newer and more efficient methods have been developed but the challenge of moving from the bench to the factory remains. Shim continues;

After the clinical proof of monoclonal antibodies as a valid therapeutic modality in 1980s and 1990s, efforts to improve the efficacy and broaden the mode of action of therapeutic antibodies have led to the successful development of gemtuzumab ozogamicin and catumaxomab. These early examples of ADC and bsAb, respectively, were later withdrawn from the market in part due to limited efficacy and/or excessive toxicity (although gemtuzumab ozogamicin was reapproved in 2017). However, advances in the antibody engineering technologies allowed the generation of safer, more efficacious ADCs and bsAbs, many of which are in commercial or late clinical development stages and discussed in this article.

In spite of the promises offered by these formats, they also pose unique technical challenges, many of which can be addressed by optimizing the production process and the physicochemical properties. However, some of these challenges are inherent to the core concepts of bsAbs or ADCs. These include balancing affinities of individual arms of bsAbs to maximize their therapeutic window, achieving synergism by bispecificity, and minimizing on-target, off-tumor toxicity of these highly potent molecules

Future developments in bsAb and ADC fields are expected to solve many of these issues to provide safer, more efficacious therapies for serious diseases with unmet medical needs. Finally,

an interesting development in the field is the combination of bsAb and ADC technologies, or bispecific antibody–drug conjugates (bsADC).

For example, a recent study reported that co-administration of HER2×PRLR bsAb with anti-HER2 ADC drastically enhanced the cytotoxic activity of the ADC, and HER2×PRLR bsADC showed a ~100-fold decrease in EC50 against the T47D/HER2 cell line relative to anti-HER2 ADC (0.4 nM vs. 40 nM, respectively) , due to the rapid internalization and lysosomal trafficking of PRLR that leads to efficient degradation of the ADC and release of the cytotoxic payload. The amalgamation of technological advancements in bsAb and ADC fields, along with a better understanding of cancer and target biology, is expected to produce more innovative cancer therapeutics that can benefit patients with currently intractable diseases.

Thus there are many challenges but many opportunities. We examine a few here. These are just samples and we have listed many other cancer targets earlier.

7.1 PROSTATE CANCER

Prostate cancer (PCa) is a significant disease in the modern world[12]. Several therapeutic approaches have been made but the complexity of PCa makes any single targeted approach limited. As Heitmann et al note:

The bispecific PSMAxCD3 antibody CC-1 is an optimised IgG-like molecule (IgGsc format) with substantially improved serum half-life, especially when compared with the prototypical BiTE bsAb format. Specific modifications introduced in this proprietary format further reduce aggregation tendency and thus unspecific 'off-target' T cell activation and immunogenicity.

Its target is, in prostate carcinoma, expressed on both, tumour cells and tumour vessels. Vascular expression is expected to facilitate access of immune effector cells to the tumour site. Notably, CC-1 binds a unique PSMA epitope which allows for such dual targeting not only in prostate carcinoma, but also in squamous cell carcinoma of the lung (data provided in the patent application)…

CC-1 is developed in a novel IgG-like format termed IgGsc to overcome several problems of so far available bsAb constructs: drawbacks in particular of bsAbs in the BiTE format are their low serum half-life (approximately 1hour) and aggregation tendency, which necessitates cumbersome application protocols and results in offtarget activation of T cells, respectively. Besides blinatumomab, this also holds true for the BiTE PSMAxCD3 bsAb developed by Amgen that presently is undergoing evaluation in phase I studies.

The IgGsc format of CC-1 not only allows for a longer serum half-life, but also has a lower aggregation tendency compared with the prototypical BiTe format, with accordingly reduced off-target T cell activation and thus fewer side effects. In addition, to achieve our superordinate goal, the safe application of sufficiently high bsAb doses which in turn shall facilitate better

[12] https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach

clinical efficacy, we will employ pre-emptive IL-6R blockade to prevent rather than to treat CRS. The prophylactic application of tocilizumab should abolish the clinical effects of CRS without impairing T cell antitumour reactivity. An additional advantage of CC-1 is that its target antigen PSMA is expressed on prostate carcinoma cells as well as on the tumour vessels of CRPC.

As noted, PCa requires multiple modes of targeting. Thus, the authors continue:

Thereby, a dual mode of anticancer action is enabled: targeting the tumour vessels should allow for improved influx of T cells into the tumour via the damaged endothelial barrier followed by effective combating of the tumour cells themselves. Thereby we hope to overcome a critical factor that so far limits the success not only of bsAbs, but of T cell-based treatment of solid tumours in general. Taking into account the lack of effective treatment options and the dismal prognosis in the study patient population, the expected benefits of a CC-1 treatment with pre-emptive tocilizumab application in this clinical study outweigh the potential risks for the patients, especially since multiple risk mitigation measures have been implemented.

The progress and safety data will be monitored by three independent experts (DSMB) and they will give approval/recommendations to the coordinating investigator/the sponsor whether to stop the trial or to change the trial protocol. In addition, the implemented intraindividual escalation of the applied CC-1 dose constitutes an additional hallmark for patients in this trial.

Usually, early clinical trials study safety and tolerability of new drugs, with therapeutic benefit for patients accordingly being only of secondary interest. In our view, this constitutes an ethical dilemma, which we tried to resolve by our approach to rapidly increase CC-1 dose levels and thus reach doses levels that were preclinically effective already for the first patients treated. The concomitant prophylactic application of tocilizumab further supports the feasibility of the fast, intra-individual dose escalation and in turn the rapid evaluation of the MTD of CC-1 as first step. In case that no DLT is observed, already the fourth patient will receive the target dose. After six further patients that receive the maximum test dose, the dose escalation part would be completed, which constitutes an important particularity of our trial.

The dose escalation phase is then followed by a dose expansion phase (also with prophylactic IL-6R blockade), as this approach has been shown to be efficient and beneficial for patients in early clinical trials.

Immunotherapy is an expanding area for the treatment of various cancers. Ipilimumab was one of the early approaches to melanoma, what was thought to be an insurmountable malignancy. It did provide some efficacy. Now as Handa et al note:

An exciting and upcoming focus in immunotherapy is the production of genetically engineered Bi-specific antibodies, which serve not only as an effective link between the attacker (e.g., CTLs or radionuclides) and the target (e.g., cancer cells) but also interrupt two distinct oncogenic mediators. In a pilot phaseI study, eight mCRPC patients received CTLs along with antiCD3 x anti-Her2 bispecific antibody (Her2Bi) and low dose IL-2 plus GM-CSF. One patient showed partial response, while three out of seven patients had a substantial PSA decline as well as a significantly improved subjective assessment of pain. Objective evaluation showed a rise in the

levels of interferon gamma (IFN-γ) and Thelper cell type 1 (TH-1) cytokines in peripheral blood mononuclear cells of two participants post treatment.

With these encouraging results in mind, a phase II trial of pembrolizumab and HER2Bi armed ATCs in mCRPC patients is ongoing. ***Another class of bispecific Abs, called BiTE or bispecific T cell engagers, has been developed to target PSMA. Anti-PSMA×anti-CD3 BsAb recognize CD3+ T cells and tumor cells expressing PSMA, promoting the cytolytic action of T cells[13]. The first-in-human phaseI clinical study of antiPSMA x anti CD-3 diabody BAY2010112 was completed recently, with results yet to be published; another trial evaluating MOR209/ES414 in mCRPC patients is currently underway.***

As with many of these therapeutics the challenge is the choice of targets.

A unique challenge in the use of antibody-based therapies that target PSMA in a mouse mode is their limited serum half-life. Antibody-based immunotherapy devised using synthetic DNA plasmids that encode a therapeutic human mAb can help overcome the problems related to short serum half-life of mAbs and the need for frequent administration. Similarly, chemical coupling with polyethylene glycol, fusion with heavy chain fragments or albumin are being tested as potential strategies to overcome the short half-life of bispecific antibodies.

Thus, immunotherapy with polyAbs is a possible pathway for in PCa.

7.2 Bladder Cancer

Bladder cancer is a significant cancer in terms of incidence, morbidity and mortality. It can become quite invasive and then the results are highly unfavorable. Multiple approaches to treatment have been taken[14]. As Ma et al note:

[13] PSMA from NIH states: *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.* https://www.ncbi.nlm.nih.gov/gene/2346

[14] See Kates et al, *To characterize immune cell expression among patients with Non-Muscle Invasive Bladder Cancer (NMIBC) treated with BCG. Experimental design: Patients with NMIBC treated with intravesical BCG were identified, and a TMA was constructed using paired pre and post-BCG bladder samples. Immunohistochemistry was performed for CD8, CD4, FoxP3, PD-L1 (SP-142 and 22C3) and PD-1. A full slide review of PD-L1+ staining tumors was performed to characterize PD-L1 and CD8 co-localization. RNAseq was performed on cored tumors from available specimens. We compared immune cell populations between BCG responders and nonresponders, and between pretreatment and postreatment tumor samples. Baseline PD-L1 staining in the BCG naive population was then validated in a separate cohort. Results: The final cohort contained 63 pretreatment NMIBC cases, including 31 BCG responders and 32 BCG non-responders. …. Conclusions: One mechanism of BCG failure may be adaptive immune resistance. Baseline tumor PD-L1 expression predicts an unfavorable response to BCG and if validated, could be used to guide therapeutic decisions.*

In the present study, we aimed to investigate whether EGFR or HER2 may serve as a target for T cellmediated immunotherapy against human bladder cancer. Expression of EGFR and HER2 was detected on the surface of bladder cancer cells, including Pumc-91 and T24 cells, and their chemotherapeutic drug-resistant counterparts. Activated T cells (ATCs) were generated from healthy PBMCs that were stimulated by the combination of anti-CD3 monoclonal antibody and anti-CD28 monoclonal antibody in the presence of interleukin-2 for 14 days.

The ATCs were then armed with chemically hetero-conjugated anti-CD3xanti-EGFR (EGFRBi-Ab) or anti-CD3xanti-HER2 (HER2Bi-Ab). The specific cytolytic activity of ATCs armed with EGFRBi-Ab or HER2Bi-Ab against human bladder cancer cells was evaluated by lactate dehydrogenase activity assays in vitro. In contrast to unarmed ATCs, EGFRBi-Ab-armed ATCs and HER2BiAb-armed ATCs showed increased cytotoxic activity against bladder cancer cells. Moreover, Bi-Ab-armed ATCs expressed higher levels of activating marker CD69 and secreted more IFN-γ, TNF-α and IL-2 than did unarmed ATCs. EGFRBi-Abor HER2Bi-Ab-armed ATCs may provide a promising immunotherapy for bladder cancer…

Bladder cancer is a significant cancer in terms of incidence, morbidity and mortality. It can become quite invasive and then the results are highly unfavorable. As Ma et al note:

Administration of Bi-Abs is also a strategy for enduring T-cell antitumour potency. Recently, phase I clinical trials with ***HER2Bi-Ab armed activated T cells (ATCs) have shown encouraging results in women with metastatic breast cancer and in men with metastatic castrate-resistant prostate cancer****.*

In the present study, ***ATCs were armed with Bi-Abs to target bladder cancer cells and their chemotherapeutic drugresistant counterparts. Human bladder urothelial carcinoma cells were demonstrated to express high levels of EGFR and HER2 protein.***

A clinically approved anti-CD3 antibody was chemically conjugated with either an anti-EGFR or anti-HER2 antibodies. The anti-CD3xanti-EGFR bispecific antibody (EGFRBi-Ab) or anti-CD3xanti-HER2 bispecific antibody (HER2Bi-Ab) was then used to direct the ATCs to kill bladder cancer cells and their chemotherapeutic drug-resistant counterparts….

Note the attempt at targeting growth receptors as well as other targets. At this stage the challenge is and most likely will continue to be the determination of the proper set of targets. The use of the ATCs is a means to an end using the immune system to perform the remediation. However the putative added benefit of blocking growth receptors may be following it down an alternative suppression path.

The present study demonstrated that compared with control unarmed ATCs, those armed with either EGFRBi-Ab or HER2Bi-Ab released high levels of LDH, indicating that armed ATCs mediated specific cytotoxicity against bladder cancer cells. These results showed that T-cell cytotoxicity was dependent upon the engagement of EGFR or HER2 via Bi-Ab linkage. Indeed, at the E/T ratio of 10:1, 50 ng Bi-Ab per 106 ATCs showed remarkable LDH release against tumour cells after an 18-h incubation, whereas anti-EGFR/anti-HER2 antibody had no

inhibitory effect at the concentration of 10 µg/ml after a 72-h incubation (data not shown). Moreover, the present study supported that arming ATCs with Bi-Abs circumvented the requirement for major histocompatibility complex antigen recognition by ATCs.

***HER2Bi-Ab-armed ATCs could not kill CD3-HER2- K562 cells, demonstrating the specificity of the HER2Bi-Ab**.*

Moreover, effector cells armed with EGFRBi-Ab, but not with HER2Bi-Ab could kill EGFR+HER2- u87-MG cells.

Our results showed that ATCs armed with either EGFRBi-Ab or HER2Bi-Ab released high levels of IFN-γ TNF-α and IL-2 upon incubation with bladder cancer cells. The increased cytokine secretion indicated that the ATCs were reactivated when encountering tumour cells. IFN-γ and TNF-α secreted by Bi-Ab-armed ATCs are directly tumouricidal and can also activate endogenous immune cells in vivo, counteracting tumour-derived suppression.

Again, this is still at the stage of investigative approaches. However, from other adjunct research we know that some of these approaches are efficacious.

Moreover, based on flow cytometry assays, both HER2Bi-Ab-armed ATCs and EGFRBi-Ab-armed ATCs expressed higher levels of CD69 than unarmed-ATC counterparts. CD69, a marker of early T-cell activation, acts as a costimulatory molecule enhancing T-cell responses following TCR-ligand interaction. Our real-time images also revealed that armed ATCs but not unarmed control ATCs aggregated with bladder cancer cells in culture, clustering around the edge of the target cell bulk, indicating the specific activation of EGFRBi-Ab- or HER2BiAb-armed ATCs.

In several phase I clinical trials, patients infused with HER2Bi-Ab-armed ATCs exhibited elevated levels of cytokines in their serum, suggesting that armed ATC administration stimulated the endogenous immunity to develop antitumour activity. In conclusion, both EGFR and HER2 appear to be suitable targets for T cell-mediated immunotherapy against bladder cancer including chemotherapeutic drug-resistant bladder cancer. EGFRBi-Ab- or HER2Bi-Ab-armed ATCs may provide a promising approach for bladder cancer in the future.

The above demonstrates some potential for this cancer.

7.3 HEMATOLOGICAL MALIGNANCIES

Hematological cancers have shown significant responses to a variety of immunotherapeutic options. As Duell et al note:

Interest in the therapeutic use of bispecific antibodies was invigorated by the success of blinatumomab in patients with ALL. Bispecific T-cell engager (BiTE) antibody constructs are a class of therapeutic antibodies that are made from two single-chain variable fragments (scFv) combined into a single protein chain, and simultaneously target CD3 in the T-cell receptor complex and a tumor antigen on cancer cells. Blinatumomab, for example, consists of an anti-

CD19 scFv in the light chain variable domain (VL)- heavy chain variable domain (VH) orientation linked through a G4S linker to an anti-CD3 scFv in the VH-VL orientation.

The binding of the BiTE antibody to both the T-cell receptor and tumor antigen leads to the creation of a cytolytic immunologic synapse only with monovalent engagement of the T-cell receptor complex, which prevents the systemic activation of effector cells in the absence of target cells. Typically, to ensure T cells are not triggered in the absence of target, the affinity of the monovalent antibody arm targeting CD3 is designed to be low (in the nM range), whereas the affinity of the antibody targeting the tumor antigen is typically higher and varies depending on the tumor target.

The cytolytic synapses formed by BiTE antibodies are essentially identical in structure and composition to typical synapses created by matching T-cell receptor, peptide antigen, and MHC class I molecules. Following synapse formation, polyclonal T-cell activation and expansion results in target cell destruction through the action of lytic granules and cytokines released in the synapse, without need for antigen recognition by the T-cell receptor.

Due to its small size (approximately 54 kDa), the half-life of blinatumomab is about 1.25 hours and, as a result, it is administered daily by continuous intravenous infusion at a constant flow rate (after an initial dose escalation) in repeated four-week cycles.

The short lifetime of some of these polyAbs seems to be a common issue thus requiring near continuous perfusion.

…Dual-affinity retargeting The dual-affinity retargeting (DART) bispecific antibody platform format has some similarity to the BiTE format in that it is also a single-chain–based format.

The heavy chain of one arm is linked to the light chain of a second arm, which reduces the constraint of intervening linker sequences to achieve an association that is more like that of an IgG molecule. The two arms maintain the covalent linkage between each other, ensuring stability of the molecule. Compared with a single-chain (BiTE) bispecific antibody with identical CD3 and CD19 antibody Fv sequences, DART molecules have been shown to be more potent in the lysis of B cells.

In freshly isolated, resting human PBMCs (peripheral blood mononuclear cells), ***the cytotoxicity of the DART was found to be greater than that of the BiTE, and the concentration needed to cause 50% of maximal activity (EC50) was up to 60- times lower****.*

The enhanced killing activity was not associated with an increase in nonspecific activation of T cells or lysis of CD19-negative (CD19−) cells. The architecture of DART molecules allows the maintenance of contact between cells, which could help explain and contribute to the high level of target cell death.

They continue:

Much focus has also been directed into ***engineering additional components into bispecific antibody approaches, for example directing immune cells other than T cells to the tumor environment.***

The potent cytotoxic effector NK cell holds promise to be effectively utilized for immunotherapy, but a big challenge for NK cells in cancer immunotherapy has been the maintenance of NKcell numbers and function in vivo and the development of methods to improve their specificity for tumors.

We have seen a multiplicity of therapeutic approaches utilizing NK cells rather than just T cells or CTLs. NK cells can be very powerful but at the same time we have seen frequent cytokine storm effects[15]. Thus some balanced control is required.

However, approaches are now emerging to take advantage of NK cells. Bispecific killer cell engagers (BiKEs) and the trispecific killer cell engagers (TriKEs), were developed to better target NK cells to malignant targets*.*

BiKEs are composed of two antibody fragments, one that recognizes a tumor antigen and another directed against CD16 on NK cells*.*

Importantly, in TriKEs, the integration of interleukin (IL)-15 drives expansion of NK cells that engage with the tumor target.

It is suggested that activation of NK cells with paracrine IL-15 may reduce systemic effects compared with systemic administration of IL-15. Recent preclinical data support various advantages of TriKEs over BiKEs. For example, in an assay assessing the killing of CD33+HL-60 leukemia cells by normal donor peripheral blood mononuclear cell PBMCs, increased NK cell–mediated killing was observed with the 161533 TriKE compared with the 1633 BiKE. The TriKE also displayed the ability to restore the activity of NK cells against primary AML targets and induced NK cell proliferation. Moreover, in a murine xenograft HL-60-Luc tumor model, greater antitumor activity and in vivo persistence of human NK cells was observed with the TriKE compared with the BiKE.

In myelodysplastic syndrome[16], increased levels of myeloid-derived suppressor cells bearing a high expression of CD155 suppress NK-cell function through engagement with T-cell immunoreceptor with Ig and tyrosine-based inhibition motif (ITIM) domains (TIGIT), a negative regulatory checkpoint expressed on NK cells in myelodysplastic syndrome.

[15] https://www.researchgate.net/publication/340607207_IL-6_COVID-19_Cytokine_Storms_and_Galen

[16] see the papers:
https://www.researchgate.net/publication/280627292_MDS_METHYLATION_AND_THE_EPIGENETIC_PARADIGM and
https://www.researchgate.net/publication/280627289_MDS_PATHWAYS_AND_DNMT1_CONTROL

Although IL-15 is known to enhance NK-cell survival and stimulate activation and proliferation,30 soluble recombinant IL-15 also induces the expression of the inhibitory checkpoint TIGIT on NK cells in vitro.31 However, when IL-15 was presented in the form of the 161533 TriKE, an anti-CD16–IL-15-anti-CD33 molecule, TIGIT expression was not induced on NK cells.31 The data are encouraging and indicate that this first-of-its-kind single-chain TriKE can enhance NK-cell killing without provoking the expression of inhibitory checkpoints. This approach may lead the way for additional modifications that provide important costimulatory or agonistic stimulation to desired effector cells. The TriKE platform also has the advantage of being easily adapted to target different tumors of choice by switching the scFv portion to a specific tumor antigen

Duell et al present the following summary Table (as modified):[17]

[17] *ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ASM, advanced systemic mastocytosis; BCMA, B-cell maturation antigen; BEAT, bispecific engagement by antibodies on the T-cell receptor; BiTE, bispecific T-cell engager; BPCDN, blastic plasmacytoid dendritic cell neoplasm; bsmAb, bispecific monoclonal antibody; CL, cutaneous lymphoma; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia, DART, dual affinity retargeting; HL, Hodgkin's lymphoma; IgG, immunoglobulin G; MDS, myelodysplastic syndromes; MM, multiple myeloma; MOA, mechanism of action; NHL, non-Hodgkin's lymphoma; TandAb, tandem diabodies; TriKE, trispecific killer engager. aIgG assembled from half-antibodies.*

Format	Molecule	M0A	Targets	Condition	Developer
BiTE	AMG420 (BI 836909)	T‧cell recruitment	BCMA + CD3	MM	Boehringer Ingelheim. Amgen (Micromet)
	AMG330	T-cell recruitment	CD33 + CD3	A ⅟⅟‧L	Amgen (Micromet)
TandAb	AFM11	T-cell recruitment	CD19 + CD3	NHL. ALL	Affimed
	AFM13	Immune cell recruitment	CD30 + CD16	HL	Affimed
	AMV564	T-cell recruitment	CD33 + CD3	MDS, AML	Amphivena Therapeutics
DART	MGD006 S80880	T-cell recruitment	CD123 + CD3	AML. MDS	Macrogenics. Servier
	MDG011 JNJ-64052781	T-cell recruitment	CD19 + CD3	B-cell malignancies	Macrogenics. Johnson & Johnson
TriKE	161533	NK-cell recruitment and MDSC inhibition	CD16 + CD33 with IL-15 crosslinker	MDS. AML. ASM	Oxis Biotech
cLC-hetero-H-chain IgG	MCLA117	T-cell recruitment	CLEC12A + CD3	AML	Merus N.V.
	REGN1979	T-cell recruitment	CD20 + CD3	NHL. HL. CLL	Regeneron
bsmAb[a]	RG7828. BTCT 4465A	T-cell recruitment	CD20 + CD3	NHL. CLL	Genentech
	JNJ 63709178 Duobody	T-cell recruitment	CD123 + CD3	A ⅟‧. IL	Janssen. Genmab
	JNJ-64007957 Duobody	T-cell recruitment	BCMA + CD3	MM	Janssen. Genmab
	PF-06863135	T-cell recruitment	BCMA + CD3	MM	Pfizer
scFv-Fc-(Fab) - fusions	Xmabl4045	T-cell recruitment	CD123 + CD3	AML. B-cell ALL. BPDCN. CML	Xencor, Novartis
GEMoaB	GEM333	T-cell recruitment	CD33 + T cells	A ⅟‧. IL	GEMoaB Monoclonals
BEAT	GBR1342	T-cell recruitment	CD38 + CD3	MM	Glenmark Pharmaceuticals

7.4 COVID-19 VIRUS

COVID-19 is the China sourced corona virus which has become a pandemic outside of China[18]. As Dong et al have recently noted:

SARS-CoV-2 is a newly emergent coronavirus, which has adversely impacted human health and has led to the COVID-19 pandemic. There is an unmet need to develop therapies against SARS-CoV-2 due to its severity and lack of treatment options. A promising approach to combat COVID-19 is through the neutralization of SARS-CoV-2 by therapeutic antibodies.

Previously, we described a strategy to rapidly identify and generate llama nanobodies (VHH) from naïve and synthetic humanized VHH phage libraries that specifically bind the S1 SARS-CoV-2 spike protein, and block the interaction with the human ACE2 receptor. In this

[18] https://www.researchgate.net/publication/345813274_COVID-19_Vaccine_An_Update_and_Primer and https://www.researchgate.net/publication/340607207_IL-6_COVID-19_Cytokine_Storms_and_Galen and https://www.telmarc.com/Documents/White%20Papers/173Corona.pdf

study we used computer-aided design to construct multi-specific VHH antibodies fused to human IgG1 Fc domains based on the epitope predictions for leading VHHs.

The resulting tri-specific VHH-Fc antibodies show more potent S1 binding, S1/ACE2 blocking, and SARS-CoV-2 pseudovirus neutralization than the bi-specific VHH-Fcs or combination of individual monoclonal VHH-Fcs. Furthermore, protein stability analysis of the VHH-Fcs shows favorable developability features, which enable them to be quickly and successfully developed into therapeutics against COVID-19. …

The trimeric spike (S) proteins that protrude through the envelope of the SARS-CoV-2 virion mediate virus entry into the host cells by interacting with the human ACE2 receptor. Therefore, a major target for anti-SARSCoV-2 neutralizing antibodies in development are to block the interaction of SARS-CoV-2 S1 protein with ACE2. In particular, two popular strategies have been employed to discover and develop monoclonal IgG antibodies that can recognize SARS-CoV-2 S1 protein mainly by binding to its receptor binding domain (RBD).

We have argued this approach early on[19]. Also we have examined vaccine approaches that are based upon similar designs[20].

The first commonly used method is to clone the antibody V genes from the B cells of surviving COVID-19 patients who have mounted a natural immune response against SARS-CoV-2. This strategy has yielded a number of neutralizing monoclonal antibodies; however, it is important to note that the patients' antibody repertoire condition and the timing of blood sample collection play a critical role in its success. The other well-recognized and classic approach for antibody generation is by immunizing humanized mice.

Additionally, new SARS-CoV-2 antibodies were developed by screening cross-neutralizing antibodies for the SARS-CoV-2 S1 protein binders from the antibodies that were initially tested or developed to treat SARS by blocking SARS-CoV S/ACE2 or MERS by blocking MERS-CoV S/CD26 interactions. One of the cross-binders is a single domain antibody/ nanobody (VHH) generated from SARS-CoV S-immunized llama. Moreover, VHHs against SARS-CoV-2 have also been generated from the llama VHH libraries. The approach of using camelid antibody VHHs is advantageous because the VHH regions are easy to produce, are stable, and are smaller sized, which increases the possibility to target unique epitopes that are not accessible to conventional VH/VL antibodies

Thus, the above polyAb approach is therapeutic rather than preventative.

[19] https://www.researchgate.net/publication/340607207_IL-6_COVID-19_Cytokine_Storms_and_Galen

[20] https://www.researchgate.net/publication/345813274_COVID-19_Vaccine_An_Update_and_Primer

8 OBSERVATIONS

Based upon the discussion above we have several observations worth noting.

8.1 PRODUCTION AND SCALING

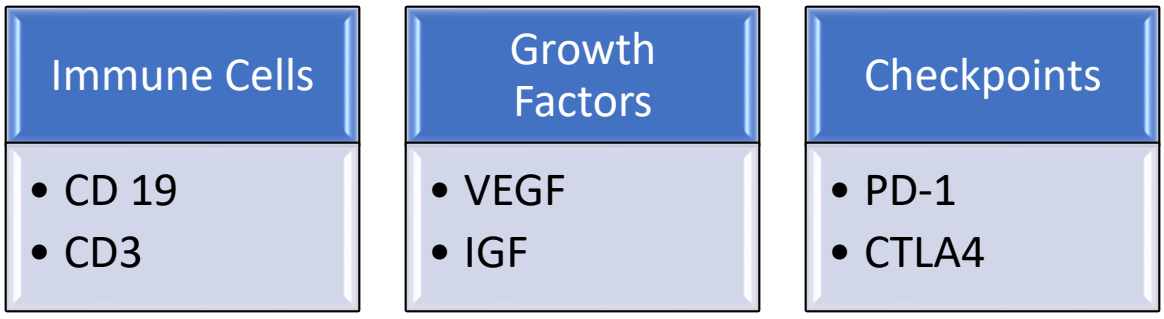
The production and scaling of polyAb is highly complex and we have presented the observation of many researchers in this area. MAbs have achieved a reasonable level of production and scalability after almost two full decades. We anticipate polyAb to do the same. For example, Quadroma technology is fairly inefficient. The reason sees somewhat obvious because we end up producing a multiplicity of configurations and seek to isolate only one of that much larger number. This dramatically reduces yield and even worse creates a massive amount of biological waste.

The papers by Andersson et al and Wang et al present details of current production methods. We have used the Andersson et al report as a substantial part of our Appendix which presents summaries of the production methods.

Issue such as yield, scaling, quality control, and waste management are a critical driver of the ultimate costs and viability of these therapeutics. We expect significant improvements in this area.

8.2 TARGETING

Targeting Ag and other proteins is a critical factor. We have given multiple examples and there have been others over the past decade or so. Checkpoint inhibitors such as PD-1, PD-L1, CTLA4 have been a cornerstone in cancers. Blocking of multiple growth factors can inhibit a variety of intracellular pathway dynamics[21]. Activating immune system cells is also a key approach.



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

In addition we have the potential of the polyAb being a carrier of a therapeutic to a specific cell. In this case the paper by Shim is an excellent summary. Specifically Shim notes:

In order to overcome the limitations of therapeutic antibodies and enhance their efficacy, various engineering and modification approaches have been devised and applied to the conventional immunoglobulin molecular format. Arguably the most prominent of these approaches are the bispecific antibody (bsAb) and antibody–drug conjugate (ADC) formats.

The basic ideas behind these formats are quite straightforward: For bsAb, simultaneous engagement of two different targets by a single antibody-like molecule may have synergistic or emergent therapeutic effects, and for ADC, the cancer-selective delivery of potent cytotoxic payloads may eradicate target-expressing cancer cells while sparing normal healthy tissues. However, the implementation and clinical application of these novel formats require a considerable amount of molecular engineering efforts.

Antibody physicochemical properties such as solubility, propensity for oligomerization/aggregation, and thermal and chemical stability, as well as their pharmacological characteristics in vivo, are all affected by combining two antibodies or their fragments, or by attaching cytotoxic payloads through chemical linkers.

Shim presents the following Table (as modified) recounting the use as a therapeutic targeting entity:

Bispecifics			
INN	***Technology***	***Target***	***Indication***
Blinatumomab	BiTE	CD19/CD3	B-cell ALL
Catumaxomab	Quadroma	EpCAM/CD3	Malignant ascites
Emicizumab	Common LC	FIXa/FX	Hemophilia
AFM11	TandAb	CD19/CD3	ALL
Duvortuxizumab	DART	CD19/CD3	B cell
ABT-165	DVD-Ig	DLL4/VEGF	Solid tumor
Vanucizumab	CrossMab	Ang-2/VEGF	mCRC
Faricimab	CrossMab	Ang-2/VEGF	AMD
JNJ63709178	DuoBody	CD123/CD3	AML
JNJ61186372	Duo Body	EGFR/cMET	NSCLC
Ab-Drug Conjugate			
INN	***Linker Payload***	***Target***	***Indication***
Gemtuzumab ozogamicin	hydrazone-calicheamicin	CD33	AML
Trastuzumab emtansine	SMCC-DMl	HER2	Breast cancer
Brentuximab vedotin	vc-MMAE	CD30	HL, ALCL
Inotuzumab ozogamicin	hydrazone-calicheamicin	CD22	ALL
Polatuzumab vedotin	vc-MMAE	CD79b	DMBLC
Enfortumab vedotin	vc-MMAE	Nectin-4	mUC
Trastuzumab deruxtecan	ggfg-MMAE	HER2	Breast cancer
Anetumab ravtansine	SPDB-DM4	Mesothelin	Mesothelioma
Depatuxizumab mafodotin	mc-MMAF	EGFR	Solid tumors
soravtansine	SulfoSPDB-DM4	FOLRa	Ovarian cancer
Rovalpituzumab Tesirine	va-SG3199	DLL3	Solid tumors

The papers by Litek et al and by Bot et al are details regarding target selection. The paper by Norman et al presents a multiple set of computational tools to address this issue as well. Targeting will become an ever more complex plays between understanding cellular dynamics as well as cellular environmental interactions.

Fundamentally, the problem of targeting is the ultimate challenge. We have extremes. On the one hand we want to utilize the immune system itself. In that class we have the extremes of NK or innate elements or T cells and adaptive elements. The second approach is pathway control. Here we may seek to block various growth factor receptors[22]. There are multiple other approaches that

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

focus on tumor microenvironments, tumor associated macrophages[23], TEM[24], fibroblasts[25] and the like.

8.3 IMMUNE RESPONSES

Cytokine storms are now a well-known after effect of uncontrolled immune responses[26]. It is not at all clear how the immune system will respond to all of these new AB/Ag interfaces. We have seen in CAR-T cells that on the one hand we obtain excellent responses to attacking the malignancy bot on the other hand we do have the potential for deadly cytokine over reactions[27].

8.4 COMPARISONS

The direction of development will depend upon a multiplicity of factors. We use the Table (as modified) from Duell et al below to illuminate some of these features:

[23] https://www.researchgate.net/publication/336116071_Tumor_Associated_Immune_Cells_On_the_one_hand_and_on_the_other_hand

[24] https://www.researchgate.net/publication/330222973_EMT_and_Cancers

[25] https://www.researchgate.net/publication/341788660_Fibroblasts_and_Cancer_The_Wound_That_Would_Not_Heal

[26] https://www.researchgate.net/publication/340607207_IL-6_COVID-19_Cytokine_Storms_and_Galen

[27] https://www.researchgate.net/publication/309419224_CAR_T_Cells_and_Cancer

Properties	Bispecific antibodies	Monoclonal antibodies	Antibody-drug conjugates	CAR T cells
Cytotoxicity	Mediated by redirecting immune cells to the tumor	Mediated by Fc$^{\gamma}$/ receptor activation on effector cells or by blocking action of growth factors	Mediated by cytotoxic payload attached to antibody	Patients' own T cells are modified to bind to antigen on cancer cells and kill them
Molecular weight	Few kDa to 1,000 kDa	Few kDa to 150 kDa	Few kDa to 1,000 kDa	Not applicable
Serum half-life	Varies from hours to days	Days to weeks	Days[72]	Weeks – years
Dosing regimen	Weekly to monthly cycles	Varying dosage and dosing regimens. Ranges from weekly to monthly to six months	Weekly to monthly cycles	Typically single administration. Can be split over multiple injections (e.g., three injections, each one day apart)
Common toxicities	Neutropenia, infections, severe cytokine release syndrome and neurological symptoms	Hives or itching, chills, fatigue, fever, muscle ache, nausea, vomiting, diarrhea, skin rash, hypotension	Anemia, neutropenia, peripheral neuropathy, thrombocytopenia, hepatic toxicity, ocular toxicity	Cytokine release syndrome, tumor lysis syndrome, neurotoxicity

9 APPENDIX – POLY SPECIFIC OPTIONS PRODUCTION

In this Appendix we provide a summary of production issues for a wide variety of poly-specifics[28]. Wang et al conclude in their presentation regarding production:

This review has focused on the design, production, and quality of bispecific antibodies. A key challenge is how to produce uniform bispecific antibody with high quality and limited or negligible side products and impurities. For scFv-type bispecifics, the protein stability and tissue penetration ability vary and depend on different types of scFv antibody.

Furthermore, with multiple host options to choose from, the determination of the most suitable system depends on the specific scFv antibody size, amino acid sequence, protein conformation, solubility, stability, purification, and scalability. For IgG-like full-size bispecific antibody, the production of pure heterodimer is achieved by complete heavy chain and light-chain heterodimerizations. Knobs-into holes method is an efficient means with which to associate different heavy chains. The common light chain and CrossMab technology are also useful approaches for varying light chain and heavy-chain assembly.

More recently, co-culture and cell-free systems are also emerging as complementary production platforms to generate bispecific antibodies readily. Advanced protein and production engineering technologies in the antibody field have boosted the development of bispecific antibodies and their derivatives, which represent one of the fastest-growing next-generation of antibody therapeutics. Diversity has been obtained in the bispecific antibody structure design both in the scFv- and IgG-like formats or by using a combination of both.

Furthermore, the addition of small molecules such as aptamers, affibodies, and synthetic drugs can further expand their applicability, creating a plethora of novel bispecific antibody-related products . Bispecific antibodies have found wide applicability to immunotherapy for cancer treatment, and these diverse molecules have the potential to treat other diseases, such as infections, acquired immune deficiency syndrome (AIDS) and genetic diseases as well as serving for medical diagnosis purposes. Looking forward, with continuous efforts to improve their design, production, and purification on an industrial scale, bispecific antibodies will represent an increasing share of the therapeutics in the market with the capacity to reach their full potential as a complementary approach to the conventional therapy in the next decade.

9.1 FC BASED FORMATS

9.1.1 Dual Variable Domains Ig (DVD-Ig)

[28] All sections herein are referenced from the work by Andersson et al. They are all in quotes and have been taken from their report.

…the heavy and light chains that compose the variable domain for binding to DLL4 and VEGF was synthesized with two-step PCR. Known domain sequences from humans were used to design the chains. Primers were designed with flanking regions to the cloning vector as well as a linker region between each variable domain. These were inserted into a vector and positive cloning vectors were identified through bacterial transformation. After being harvested and purified, the vector encoding genes for the recombinant bispecific binding protein were expressed via mammalian host cells.

Affinity chromatography is used for purification, preferably protein A chromatography. Here, the Fc region is utilized to capture the DVD-Ig. …suitable resin for this matter is MabSelect or MabSelect SuRe from GE Healthcare or ProSep Ultra Plus from EMD Millipore. The purification of a DVD-Ig may include further steps of ion exchange chromatography and/or hydrophobic interaction chromatography as well. Such steps may be anion exchange chromatography, mixed mode chromatography of either cation exchange or anion exchange type, hydrophobic interaction chromatography and viral filtration.

9.1.2 scFv-Ig Fusions

The production of these formats is, because of their symmetric design, easy and standard processes used in the production of antibody fragments and IgG-like antibodies can be applied. Because they contain both Fc region and scFvs, it is assumed that protein A, G and protein L can be used.

9.1.3 scFv-Fc Fusions

9.1.4 DART

A bispecific antibody of this format can be produced by fusion of two different binding domains, each to a separate Fc chain. Any problems with impurities can then be avoided by applying one of the existing strategies that drives heterodimerization. For example, DART-Fc antibodies can be generated by fusing one or more DART fragments to one Fc that contains the KIH substitutions. This design enables purification with protein A and G.

9.1.5 BEAT

The BEAT antibodies were produced by using following production steps. Preparing DNA expression vectors using standard molecular technology. This can be made by preparing three expression constructs, one for the scFv-Fc fusion, one for the heavy chain and one expressing the corresponding light chain. Following, the DNA vector(s) are transfected or co-transfected into a mammalian cell line. In the patent, HEK cells were used but also CHO cells can be used according to Glenmark. The BEAT antibodies were purified using a two-step process consisting of a capture-elution mode chromatography step using protein G. This step is followed by gradient mode chromatography using protein A chromatography and pH elution. Examples of possible protein A resins that can be used in the purification is MabSelect SuRe or Mabselect protein A column but are not limited to these two.

9.1.6 XmAb

In order to construct the XmAb format an antibody heavy and light chain and a scFv Fcfusion were subcloned into vectors. The scFv and Fc region were connected with a GS-linker. The Fc region was altered with substitutions in order to increase the differences in pI between the two heavy chains. This would increase the pI differences between homodimers and heterodimers, which would then facilitate the purification of heterodimers. They sought to minimize the risk of immunogenicity by utilizing buried substitutions, but the exact risk has to be further investigated in clinical studies. For the production of the proteins, plasmids encoding all chains were co-transfected into HEK cells. The antibody was purified using protein A chromatography and ion exchange chromatography.

9.2 FRAGMENT BASED

9.2.1 BiTE

In a study by Naddafi et al. (2018), the upstream and downstream process of blinatumomab was described. Both CHO cells and Escherichia coli (E. coli) strains where tested as expression hosts for the upstream process. The gene coding for blinatumomab was cloned into expression vectors, 6xHis-tagged and purified on a Ni-NTA chromatography column. The NiNTA column is used for purification of 6xHis-tagged recombinant proteins. The result showed that the purified antibody from the CHO cell expression system had higher binding activity compared with the purified antibody from the E. coli expression system. This is due to a more properly folding of proteins in mammalian cells compared to E. coli cells.

9.2.2 TandAb

Purification by protein A or G chromatography is not possible due to the lack of an Fc region. Purification via His-tag as a capture step is a possibility and seems to be the most common capture method. Purification via protein L chromatography is an alternative as well. A bimodal polishing strategy, i.e. based on two orthogonal properties, can be performed based on purity needs. This can include size exclusion chromatography together with protein A or G chromatography, where fragment-based antibody formats can be collected as flow-through.

9.2.3 biAbFabL

Hybridoma cells were used to produce parental antibodies. To design a humanized antibody, murine complementarity-determining regions were grafted onto human germline framework sequences for both heavy and light chains. Humanizing the antibody is key to reduce the immunogenicity of the antibody, which is a factor to be wary of when developing biological therapeutics. They also used a modified version of IgG called IgG1.1 with reduced FcR binding ability to avoid the binding of phagocytosis-mediating receptors. This would also reduce the immunogenicity of the format.

The bispecific antibodies were constructed via genetic fusion of the parental antibody sequences by using a HEK transient expression vector, with inserted genes for the chains using restriction

enzymes. The vectors were transfected into E. coli for amplification. After harvesting and purification, HEK suspension cells were transfected with expression constructs using polyethylenimine and then cultivated. Media was then harvested. Cells were lysed using ultrafiltration. Protein was purified using a bimodal strategy consisting of a capture step using MabSelect SuRe (GE Healthcare) and then a polishing step using Superdex SEC (GE Healthcare). Since all of the formats contain the Fc region, using protein A affinity chromatography as a capture seems natural. The appended binding entities deviating the structure from native IgGlike form does not appear to hinder this strategy in any way. Hybridoma cells were used to produce parental antibodies.

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9.2.4 MAT-Fab

The design of a MAT-Fab antibody is based on parental monoclonal antibodies which have at least one property of the desired MAT-Fab antibody. Examples of properties are affinity to the wanted antigen, stability or solubility. A parent antibody may thus not have affinity to the wanted antigen if the derived part from it e.g. is a constant domain. The constant domain must in that case be linked to a variable domain from a different parental antibody with correct affinity. The parental antibodies could be naturally occurring or genetically constructed antibodies. Production of each subunit was made by introducing vector(s) with one to four of the polypeptide chains that form the MAT-Fab into host cells.

9.2.5 iBiBody

To produce an iBiBody antibody, DNA vectors encoding one or more of the polypeptide chains the iBiBody consists of were made. This was done with multiple overlapping PCR. These vectors were then expressed in HEK. Purification was done with protein A affinity chromatography or protein G affinity chromatography. In example two mentioned in the patent, where they designed a FLT3xCD3 iBiBody, the protein A Sepharose 4 Fast Flow column chromatography from GE

Healthcare was used. In order to maintain affinity to protein A or G, two residues of the Fc portion of the iBiBody may be replaced

9.2.6 Tandem Forms

The format was produced by means of genetic fusion where the variants had HC and LC encoding sequences produced separately. The two Fabs were expressed in tandem and bound together using GS linker for the HC and either a GS linker or a polypeptide linker including a protease recognition site in order to enable LC linker cleavage. For information regarding the order of Fab regions and linker equipped for each format variant. The HC and LC were then inserted into a mutual vector. Vectors containing the chains of all constructs, to be cleaved and ligated using either the ligation kit "Ligation-Convenience Kit" (NIPPONGENE Co., Ltd.) or ligation reagent Ligation-high (TOYOBO Co., Ltd.) and were transfected in host cells, preferably CHO, HEK or NS0 cell lines, transfected with the vectors.

The culturing was carried out using established methods. Possible LC polypeptide linkers were then cleaved by intracellular proteases as to improve the binding affinity of primary antigen binding sites were then performed. The method of producing the bsAbs may include a recovering step preferably by means of isolating or purifying from the host cell line in addition to a step regarding the direct culturing of a construct expressing cell line. The bsAbs can preferably be purified from the culture supernatant by means of various chromatography methods, for example protein A or protein G columns. Although these methods are preferred, several other methods can be utilized, such as gel filtration, ultrafiltration or ion exchange chromatography.

9.2.7 κλ antibody

The κλ antibody format is also patented by Novimmune and is described in their patent application. The first step in the upstream process of the κλ antibody was to isolate two antibodies with different specificities and variable domains, but with the same variable heavy domain. The variable heavy chain domain was then fused with the constant region of the heavy chain. The two different light chain variable domains were fused to their respective kappa and lambda constant domain. Lastly, the three peptide chains were co-expressed in mammalian cells, e.g. in CHO cells. This generated three types of antibodies: two monospecific antibodies and one bispecific antibody.

Due to the kappa and lambda constant domains, affinity to these domains can be utilized in the purification process. There are three types of antibodies as mentioned - two comprising either kappa or lambda constant domains and one comprising both kappa and lambda constant domains. By using affinity to both kappa and lambda constant domains, only the bispecific antibody with both domains will remain after the purification process.

This general approach is divided into a three-step chromatography. In the first step, affinity to the Fc domain is utilized which captures the antibodies. In the second step, affinity to the constant kappa light chain is utilized, which captures antibodies with one or two kappa light chains. In the third and last step, affinity to the constant lambda light chain is utilized. This captures the bispecific kappa/lambda heterodimers. (Brinkmann & Kontermann 2017) The same

approach was described in the patent application (US009834615B2, 2017) with the following three steps: (i) Protein A that captures both mono- and bispecific IgG antibodies, followed by (ii) KappaSelect (GE Healthcare) that captures antibodies containing one or two kappa light chains, and (iii) LambdaSelect (GE healthcare) that captures antibodies containing a lambda light chain. The antibody remaining is bispecific.

9.2.8 ADAPTIR

For upstream processing of this antibody, viral and non-viral vectors are used. The nucleotide sequences encoding VH and VL for one binding domain are most commonly placed in a plasmid. The sequences are then synthesized or amplified and linked together with a polypeptide linker. The resulting scFv nucleotide sequence, particular for a certain binding domain, is fused with the sequence for an Fc region. The sequence for the second binding domain is synthesized or amplified and linked to the Fc region in the same way.

The resulting bispecific antibodies are expressed in host cells via transient transfection of cultured mammalian cells. Some suitable and commonly used cell lines for this purpose are HEK and CHO cells. Aptevo Therapeutics state that the production of ADAPTIR antibodies can be done using antibody-like processes at commercial scales (Aptevo Therapeutics, 2019). Affinity chromatography methods are used to purify these bispecific antibodies. Due to the fact that the ADAPTIR molecule contains an Fc region, the antibodies can be purified with protein A or protein G chromatography. However, protein A was used for purification of the molecules in the examples in the patent. For further purification size exclusion chromatography is also applied.

9.2.9 BiIA-SG

BiIA-SG antibodies are produced by fusing genes encoding two scFvs with different binding affinities in tandem, connected by a linker to a human IgG-Fc domain. The scFvs are connected through fusion of the gp120 binding VL/VH to the N-terminal of the CD4 binding VL/VH. The single-gene constructs are introduced into plasmids and transfected into cells. CHO cells were used for protein production in in vivo studies but HEK cells were used for the in vitro analysis. After culturing and centrifugation, the antibodies were purified using Protein-G-Agarose (Life technologies) according to the manufacturer's instructions. Purity was validated to 90% using SEC-HPLC and no extra polishing steps were mentioned.

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