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T cell engaging bispecific antibody (T-BsAb): from technology to therapeutics

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Abstract

Harnessing the power of the human immune system to treat cancer is the essence of immunotherapy. Monoclonal antibodies engage the innate immune system to destroy targeted cells. For the last 30 years, antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity have been the main mechanisms of anti-tumor action of unconjugated antibody drugs. Efforts to exploit the potentials of other immune cells, in particular T cells, culminated in the recent approval of two T cell engaging bispecific antibody (T-BsAb) drugs, thereby stimulating new efforts to accelerate similar platforms through preclinical and clinical trials. In this review, we have compiled the worldwide effort in exploring T cell engaging bispecific antibodies. Our special emphasis is on the lessons learned, with the hope to derive insights in this fast evolving field with tremendous clinical potential.

Keywords

cancer immunotherapy; bispecific antibodies; T cell engaging bispecific antibodies; potency of bispecific antibodies; TDCC; bivalent CD3 binding

1. Introduction

Cancer remains one of the leading causes of death, with the accompanying social and economic burden worldwide. While surgery is effective for locoregional control, chemotherapy and radiation have been mostly ineffective for metastatic cancers, even when pushed to dose and intensity limits, which alone can be harmful because of their inability to discriminate cancer cells from normal bystanders. To minimize toxicity, much efforts have been devoted to identify therapeutic agents that can selectively inhibit the growth of or eradicate cancer cells, while leaving normal cells unscathed – a concept dubbed the “magic bullet” by Paul Ehrlich more than 100 years ago. Before the advent of pathway-specific

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small molecule inhibitors, antibody-based drugs had been the centerpiece of these efforts and they will likely remain a major player in the coming decades in cancer therapy.

Antibodies are extraordinary molecules vetted through millions of years of evolution. Each antibody molecule has two identical antigen binding sites at the N-terminal variable region that are responsible for the exquisite antigen binding specificity and the binding affinity of these molecules, and a constant fragment crystallizable (Fc) region at the C-terminus that triggers multiple effector mechanisms (Vidarsson, Dekkers, & Rispens, 2014). Depending on the specific antigen/antibody pair, binding alone can physically block the antigen (receptor) or initiate/inhibit signaling through the antigen (receptor) leading to apoptosis of target cells. For the majority of cancer therapeutic IgG antibodies, they execute their immune functions through recruitment of natural killer cells or myeloid cells/macrophages via the Fc region. Furthermore, the Fc region can initiate the classical complement cascade to deposit membrane attack complex on the surface membrane of target cells. These Fc-dependent tumor lysis mechanisms have been extensively studied and exploited in human medicine.

Soon after the discovery of the hybridoma technique by Hans Kohler and Caesar Milstein (Kohler & Milstein, 1975) to immortalize B-cells, the first monoclonal antibody muromonab-CD3 (OKT3) specific for human CD3 was developed and approved in 1985 for treating organ transplant rejection. It took the next decade before the first cancer therapeutic antibody rituximab was approved in 1997 to treat CD20(+) non-Hodgkin lymphoma. Since then, at least 27 therapeutic antibodies for a broad spectrum of human cancers have been approved. The success of these antibody therapeutics firmly established cancer immunotherapy as the fourth modality (after surgery, chemotherapy and radiation) whereby existing defense mechanisms of the human immune system can be mobilized to specifically kill cancer cells. However, naturally occurring IgG antibodies do not have the functionality to directly engage the most efficient “killer” in the immune system, namely, the cytotoxic T lymphocyte (CTL). In order to do that, antibodies have to be engineered to include a second specificity, hence bispecific antibodies (BsAb).

The concept of bispecific antibodies dates back to the 1960s, when Alfred Nisonoff envisioned the potential of replacing one of the two identical antigen binding arms with a different antigen binding specificity (NISONOFF A, 1961; Nisonoff, Wissler, & Lipman, 1960). This concept was developed further in the 1980s to include a second specificity against T cell determinants. CTLs, like all T cells, express variable T-cell receptors (TCRs) associated with invariable CD3 subunits. Binding of TCR by cognate peptide-major histocompatibility complex (pMHC) initiates the signaling through the CD3 complex, which in turn relays the signal internally to activate T cells. By binding to the CD3 complex, CD3-binding monoclonal antibody can bypass the pMHC restriction, thereby activating polyclonal CTLs. When such CD3 binding specificity was engineered into antibodies that bind to tumor specific antigens, CTL response can be redirected to cancer cells (Perez, Hoffman, Shaw, Bluestone, & Segal, 1985; Staerz, Kanagawa, & Bevan, 1985). This strategy gave rise to a completely new class of therapeutic antibodies for cancer immunotherapy. Although it was later found that this class of antibodies could also activate

through CD3 on non-T cells, for the purpose of this review, we refer to them as T cell engaging bispecific antibody, or T-BsAb for short.

Over the past three decades a myriad of T-BsAbs have been developed (discussed below). Although the molecular details differ considerably, they are all grounded on the basic design of combining tumor antigen binding specificity and T cell binding specificity into one molecule, with or without an Fc region. To date, only two T-BsAbs, catumaxomab and blinatumomab, have been approved for clinical use in humans, as compared to the other 25 IgG based antibody drugs. The lag is largely attributed to the difficulties in protein engineering during the manufacture of these antibodies and the uncertain clinical toxicities with these novel constructs. Nevertheless, over the past 30 years, multiple molecular designs have been invented, some of which have entered clinical stages of development and many more are in preclinical testing. In this review, we have compiled all the molecular designs that have been developed so far and discussed different aspects of T-BsAbs, including molecular details of their mechanisms of action, factors that may determine their potency, as well as different challenges lying ahead. We hope to provide a timely summary of all the lessons learned that may provide insights to help T-BsAb development in the coming decades.

2. T-BsAbs developed to date

A few recent comprehensive reviews (Brinkmann & Kontermann, 2017; Kontermann & Brinkmann, 2015; Spiess, Zhai, & Carter, 2015) have summarized the various bispecific antibody designs currently under development or approved. To be consistent, this review will follow the same nomenclature they adopted whenever possible. Multiple technologies have been developed to generate human IgG-like molecules; in this review we refer to them as hIgG. Figure 1 summarizes the major formats discussed in this review.

2.1. T-BsAbs in clinical development

Table 1 summarizes all T-BsAbs that have reached clinical stages so far. Out of these 23 antibodies, blinatumomab was approved for treatment of refractory/relapse Ph(−) B-ALL and catumaxomab was approved for malignant ascites derived from EpCAM(+) carcinomas. The rest are mostly ongoing or completed phase I clinical trials, except two trifunctional antibodies, FBTA05 and ertumaxomab, which have entered phase II trials for intravenous infusion. However, both studies have since been terminated.

Besides T-BsAbs against antigens expressed by hematopoietic cells, namely, B cells (CD19, CD20, BCMA) and myeloid cells (CD33, CD123andCLEC12A), it is encouraging to note that T-BsAbs against antigens expressed by solid tumors (CEA, EpCAM, HER2, PSMA, p-Cadherin, pMHC, GPC3, GPA33) are also being tested. Results from these trials will inform future strategies to optimize T-BsAbs. CD19, CD20, EpCAM, CD33 and HER2 are clinically proven targets, as they are also targets of approved IgG drugs; whereas other targets like p-Cadherin, pMHC and GPC3 are important novel targets that have not been drugged with FDA-approved or EMA-approved antibodies.

The most common format is tandem single chain variable fragment (scFv) based on blinatumomab. However, newer formats like tandem diabody (TandAb), DART and DART-Fc, hIgG, Fab-scFv-Fc, TriFab-Fc, scFv-Fc-scFv, BEAT and TCR-αCD3 are also being investigated. The m/rIgG trifunctional format was used by the first T-BsAb approved. However, apart from immunogenicity, it was severely limited by toxicity when delivered systematically (Sebastian, et al., 2007). This is likely due to their wildtype Fc with full effector functions; and as a result it has not been widely adopted. All the molecular designs incorporate monovalent CD3 binding except for TandAb and scFv-Fc-scFv, which, at least structurally, could mediate bivalent CD3 binding. The prevalence of monovalent anti-CD3 design probably stemmed from the observation that bivalent anti-CD3 antibodies could result in activation induced T cell death (AICD) (Kuhn & Weiner, 2016) and the concerns that it might cause target independent T cell activation. However, AICD in T-BsAb will likely be platform-specific, since for at least 3 formats using bivalent anti-CD3 design, T cells seemed to be fully functional in vitro and in animal models (discussed below). Therefore, the clinical outcomes of these bivalent formats (two are currently in trial) would be informative in the future design of the optimal T-BsAb.

2.2. Preclinical T-BsAbs

The concept of T-BsAb was explored initially in 1985 in murine system using anti-mouse CD3 antibody; but within a few months the first T-BsAb using anti-human CD3 was developed (Perez, et al., 1985; Staerz, et al., 1985). The following decades saw an “explosion” of bispecific antibody development (Riethmüller, 2012). T-BsAbs engineered for human use were dominated initially by chemical conjugation of either full-length IgG or F(ab’), or by hybrid hybridoma technology. Since then, a plethora of T-BsAb formats have been described (Table 2). These include most of the formats used by non-T bispecific antibodies (Brinkmann & Kontermann, 2017; Kontermann & Brinkmann, 2015; Spiess, et al., 2015). The most frequently used format is tandem scFv (BiTE), partly because it avoids issues of cognate chain pairing in multichain constructs, and partly because of its clinical success epitomized by blinatumomab. With the advent of full length bispecific Ig formats that overcome these pairing issues (Figure 1), T-BsAbs with more native conformations can now be more easily manufactured while achieving more desirable PK-profiles than BiTEs (discussed below) and are becoming more widely adopted.

In addition to the large number of formats, more than 44 antigens have been targeted, with varying degrees of success in preclinical models. The majority of these antigens are oncogenic proteins, except for a few targets in infectious diseases which are not the focus of this review. The most commonly targeted antigens are EGFR, CD19, CD20, CD33, CEA, EpCAM and HER2, all of which have been targeted by more than one format. Peptide-MHC is an interesting class of antigens that has emerged in recent years. Traditional targets for therapeutic antibodies are expressed on cell surface, while most oncoproteins are expressed intracellularly and inaccessible to conventional antibodies. However, peptide fragments of some of these proteins generated via protein turnover can be presented by MHC on the cell surface, which greatly expand the repertoire of “druggable” targets. Immunocore Limited has pioneered the affinity maturation of TCR fused to anti-CD3 scFv. Moreover, TCR-like

therapeutic antibodies that target pMHC in a similar fashion as TCR are also emerging in the past few years and are currently actively pursued (Dao, et al., 2015).

Most T-BsAbs developed so far utilize anti-CD3 moiety for T cell recruitment. Excluding those T-BsAbs that did not disclose their anti-CD3 sequences, most of the T-BsAbs developed to date used clones derived from OKT3, UTCH1, L2K or TR66. These mouse-derived antibodies have been humanized, affinity matured or deimmunized, depending on the formats/developers. Based on the available kinetic data, the affinities of these anti-CD3 antibodies span a wide range from 1-200nM by surface plasmon resonance analysis and 8-500nM by flow cytometry analysis (The effect of anti-CD3 affinity will be discussed in the next section). Our compilation also showed that other triggering molecules like TCR, CD5 and CD2 have also been successfully used in the past. In a study using anti-CD19 antibody in a BiTE format, Moore et al. compared the effects of different T cell triggering modules and showed that there was no significant differences between anti-TCR and anti-CD3 in T-cell dependent cellular cytotoxicity (TDCC) assays (P. A. Moore, et al., 2011). Similarly, Tita-Nwa et al. also showed that CD19xCD5 from hybrid hybridoma lysed lymphoma cells with potency comparable to CD19xCD3 when activated T cells was used as effector cells, except that it did not induce resting T cell proliferation and induced less AICD (Tita-Nwa, et al., 2007). Anti-CD2 antibodies alone usually do not activate T cells; by using two anti-CD2 antibodies M1 and M2, Wild et al. demonstrated that M2xEGFR could activate TDCC in an M1-dependent manner (Wild, et al., 1999). All these results suggest that triggering molecules other than CD3 can be viable alternatives for engaging T cells.

3. Lessons learned over three decades of T-BsAb research

3.1. Mechanisms of action

The original intent of developing bispecific antibodies with anti-CD3 specificity was to recruit CTL to kill tumor cells (Staerz, et al., 1985). However, other immune cells, including γδ T cells, natural killer T cells and CD4(+) T cells, also express CD3 and in theory they all can be activated by T-BsAbs. Indeed, it has been shown that γδ T cells are as potent as CD8(+) T cells in TDCC assay by an EGFR T-BsAb (Ferrini, et al., 1993). NKT cells can be activated by anti-CD3 antibodies (Iyoda, et al., 2010) and have been shown using anti-EpCAM BiTE MT110 to have cytotoxic activity (Kischel, Hausmann, Baeuerle, & Kufer, 2009). NKT cells could be a good source of effector cells as they express invariant TCR (thus limiting potential toxicity) and can undergo robust ex vivo expansions (Heczey, et al., 2014). Both CD4(+) and CD8(+) T cells can be activated and contribute to cytotoxicity induced by T-BsAb, although CD4(+)T cells generally do so with delayed kinetics (Haas, et al., 2009). Interestingly, in a subcutaneous model of ovarian cancer, Stadler et al. analyzed the cellular composition of tumor infiltrating lymphocytes (TILs) and found that there were more CD4(+) T cells than CD8(+) T cells, consisting of T_{H17}, T_{H1} and T_{H2} subsets based on gene expression profiling (Stadler, et al., 2016). Similar results regarding the presence of both CD4(+) and CD8(+) T cell subsets in tumors have also been observed with the IgG(L)-scFv modular platform (Lopez-Albaitero, et al., 2017; H. Xu, et al., 2015). While CD8(+) CTLs perform anti-tumor effect, CD4(+) T cells also play an important role in tumor eradication, either directly or indirectly, as suggested by other studies (Matsuzaki, et al.,

2015; Quezada, et al., 2010). The relative potency of CD8(+) T cells versus CD4(+) T cells in TDCC is inconclusive in the published literature. In terms of maximal killing, some T-BsAbs (e.g., anti-CD20) induced higher maximal killing in the presence of CD8(+) T cells than that of CD4(+) T cells (Liping L. Sun, et al., 2015); whereas others (e.g., anti-BCMA, anti-PSCA and AFM11) induced similar levels of maximal cell killing for both T cell subsets (Feldmann, et al., 2012; Hipp, et al., 2017; Uwe Reusch, et al., 2015). Data regarding EC50 is more limited and equally inconclusive. Within each of the two major αβ T cell subsets, effector memory T cells appeared to be the major mediators of TDCC in the presence of EpCAM BiTE; naïve T cells on the other hand, mediated limited levels of TDCC (Dreier, et al., 2002; Kischel, et al., 2009). Interestingly, expansion of effector memory T cells following blinatumomab treatment was also associated with the anti-tumor response (Bargou, et al., 2008).

To be able to kill tumor cells, T-BsAb must be in contact with both tumor target cells and effector cells. After intravenous infusion of the antibody, it is likely that at least a proportion of the T-BsAb will bind first to the effector cells. However, the majority of T-BsAbs currently under development have a relatively fast k_{off} when binding to CD3, leading to a short residence time (dissociation $t_{1/2}$) in minutes. Whether and how this initial contact of T-BsAb with T cells can prime them to change their migration behavior likely depends on the structural design of the T-BsAb, a topic that has not been carefully investigated. One can speculate that stronger binding of CD3 through bivalency lengthens the residence time and primes T cells more efficiently. In an extreme example, catumaxomab was shown to be able to activate T cell in an antigen-independent manner (Stanglmaier, et al., 2008); and in a separate study, TNFα released from catumaxomab activated T cells could increase ICAM-1 and CD62E expression on endothelial cells to facilitate T cell adhesion (Dettmar, et al., 2012), an important step in their migration out of the vasculature. Nevertheless, most other T-BsAbs cannot activate T cells independently of target cells, at least not to the extent of catumaxomab; thus the detection of changes in T cell behavior, or lack thereof, may require more sensitive tools. Of note, catumaxomab activates T cells to such an extent that its toxicity has limited further dose escalation in human trials.

Inside solid tumor vasculature, macromolecules like T-BsAbs extravasate through transvascular pores with sizes between 200nm–1.2μm to reach tumor by diffusion (Hobbs, et al., 1998). Multiple solid tumors have been shown to contain TIL, the frequency of which is associated strongly with prognosis and tumor response to immune checkpoint inhibitors (ICI) (Gajewski, et al., 2013; Gooden, de Bock, Leffers, Daemen, & Nijman, 2011). In tumors without TILs (Spranger, 2016), they rely on the ability of T-BsAbs to recruit T cells from the blood. Under normal conditions, naïve T cells are activated by antigens in the lymph nodes and mature into effector T cells. Effector T cells then exit into the blood and migrate into tissues via a coordinated process of rolling, adhesion and transmigration, orchestrated by the sequential interactions of selectin with selectin ligand, chemokine with chemokine receptor, and integrin with adhesion molecules (Nolz, 2015). How T-BsAbs influence these steps and which subsets of T cells are recruited into tumors remain open questions. Much of the clinical experience with T-BsAbs is based on liquid tumors and malignant ascites, which do not possess the same complex architecture of solid tumors, whose vasculature can severely hinder T cell trafficking. Nevertheless, in tumor xenografts

in mice, T-BsAb was shown to be able to recruit T cells from the peritoneal cavity into tumors (Bacac, et al., 2016; Stadler, et al., 2016), or from bloodstream into solid tumor masses (Lopez-Albaitero, et al., 2017; H. Xu, et al., 2015). Whether this can be translated into efficacy in human patients will have to await future clinical trials.

Besides bringing together tumor target cells and effector cells, T-BsAbs can exert adhesive forces between the two apposing cells, as measured by atomic force microscopy (S. C. Hoffmann, Wabnitz, Samstag, Moldenhauer, & Ludwig, 2011; Seckinger, et al., 2017). T-BsAbs can induce a more stable conjugate formation between target cells and effector cells, increasing the contact time by as much as 3-fold (Bacac, et al., 2016; Salnikov, et al., 2009) and providing additional time for full activation of T cells to occur. This engagement of target cells and effector cells by T-BsAbs was shown to induce the formation of immunological synapse that is indistinguishable from the synapse formed between TCR and pMHC complex (H. Xu, et al., 2015). The basic geometry of these synapses have TCR-CD3 concentrated in the middle, LFA-1 and F-actin forming ring-like structure at the periphery, and CD45 excluded from the synapse (Griffiths, Tsun, & Stinchcombe, 2010; Li, et al., 2017; Offner, Hofmeister, Romaniuk, Kufer, & Baeuerle, 2006). Formation of immunological synapse is accompanied by the redistribution of signaling and secretory granule proteins in the cell, which eventually leads to the release of perforin and granzymes (Offner, et al., 2006). Release of perforin causes transient pore formation in the juxtaposed target cells and endocytosis of both perforin and granzyme into “gigantosomes”. Inside these enlarged endosomes, perforin again forms pores and releases granzymes into the cytoplasm to cause apoptosis of target cells (Thiery, et al., 2011). This contact-dependent cytotoxicity is likely the main mechanism for T-BsAb induced direct killing of tumor cells, as EGTA chelation of Ca^{2+}, which is required for perforin multimerization and pore formation, led to the complete inhibition of target cell apoptosis by T-BsAb (Haas, et al., 2009; Lyubchenko, Wurth, & Zweifach, 2001). Activation of T cells also results in the secretion of cytokines and T cell proliferation (Nguyen, et al., 2016), which may be required to sustain the immune reactions and their anti-tumor effects.

Although formation of immunological synapse coincides with T-BsAb binding and cytotoxicity, two molecular details have not been fully understood. First, it is unclear how monovalent anti-CD3 binding can lead to clustering of CD3 molecules on T cells. Based on the relative affinities of anti-CD19 and anti-CD3, Hoffmann et al. proposed that T-BsAb binds to target cells and serves as a T cell activation “matrix” that captures and activates mobile T cells (P. Hoffmann, et al., 2005). How this occurs mechanistically is unclear. It is possible that the close approximation of antigens leads to clustering of TCRs on T cells and their subsequent activation. Indeed, both tumor antigen and CD3 have been shown to cluster at the synapse when the target cell and T cell were brought together by T-BsAb (Blank-Voorthuis, et al., 1993; Li, et al., 2017). This is reminiscent of the effect of secondary antibody cross linking primary antibodies attached to antigen on cell surface. However, in a comparison among 3 antibodies that bind to 3 epitopes on FcRH5, it was found that the antibody which bound to the most membrane distal epitope could not induce clustering of antigen or antibody (Li, et al., 2017), arguing that simple approximation of target antigens is insufficient to activate T cells and that other factors also need to be considered, as explained in the next section. It is important to note that the binding behavior of T-BsAbs to

membrane-anchored antigens in the interface between target cells and effector cells may be very different from when they are in solution, as the antigens are constrained in two-dimensional planes, with possible boundaries set by immunological synapse (Valitutti, Coombs, & Dupré, 2010). The second unresolved molecular aspect is whether the formation of immunological synapses is absolutely required for cytotoxicity to occur. In the case of pMHC and TCR interaction, it has been shown that cytotoxicity can be uncoupled from TCR clustering and formation of mature immune synapse. This was done by the use of low concentration of pMHC that triggered maximal cytotoxicity but only minimal TCR modulation and IFNγ secretion, and the formation of rudimentary synapse. Ca^{2+} flux still occurred but displayed a spike-like pattern, in contrast to the smooth and sustained pattern observed by fully activated CTLs (Faroudi, et al., 2003). Indeed, as few as three pMHC molecules was sufficient to trigger cytotoxicity, whereas formation of mature synaspse required about ten. With three pMHC, signs of cell death could occur as early as 5-15min (Faroudi, et al., 2003; Purbhoo, Irvine, Huppa, & Davis, 2004). Interestingly, in a study where T-BsAb bound to target cell through the FcR (not through tumor antigen), T cell killing of target cells did not require TCR clustering (Blank-Voorthuis, et al., 1993). In another study that demonstrated the serial killing ability of anti-CD19 BiTE using cytotoxic T cell line MC-15, the authors did not observe any stable clustering of target cells around T cells and killing occurred in as early as a few minutes, limited seemingly by T cell movement during target cell scanning. However, formation of synapses or lack thereof was not investigated in that study (P. Hoffmann, et al., 2005). Thus, it appears that under optimal conditions (activated T cells, high effector to target ratio, and homogeneous or cloned effector cells), cytotoxicity elicited by T-BsAbs can occur with very fast kinetics, raising the possibility that it may not require the formation of mature synapses. Although further studies are required to clarify this issue, it is tempting to speculate that such mechanism will be beneficial in the diffusion front of T-BsAbs inside tumor, where antibody concentration may be low (Adams, et al., 2001).

3.2. Factors that affect the potency of therapeutic T-BsAb

Multiple factors can affect the potency of a particular T-BsAb, including the antigen itself, binding epitope, antibody affinities, and the specific format used. All these variables interact to generate a specific context that determines the efficiency of T cell activation and ensuing target cell killing. Individual variables have been investigated over the years and in the following paragraphs their importance will be reviewed.

3.2.1. Antigens and epitopes—In an elegant study, Bluemel et al. (Bluemel, et al., 2010) used a set of T-BsAbs that bind to different epitopes along the length of the melanoma antigen MCSP and compared the potency of these antibodies in TDCC assay. They found that antibodies binding to membrane proximal region of MCSP were more potent than those binding to membrane distal region. Consistently, MT110 (anti-EpCAM) gradually lost TDCC activity when the cognate antigen was artificially displaced away from the membrane by increasing number of MCSP spacer domains. In addition, this study also demonstrated that increasing the size of antigens could also block T cell cytotoxicity in the presence of T-BsAb, probably through steric hindrance that prevented T cells from accessing the target cell membrane. Using a different antigen system, Li et al. (Li, et al., 2017) similarly used a set of

three antibodies against FcRH5 to compare the effects of distance from target cell membrane on the potency of T-BsAbs. They found that only the membrane proximal antibody 1G7 efficiently caused target antigen clustering and CD45 exclusion from immunological synapse, which translated into higher potency in TDCC. On the other hand, truncating the target antigen to draw it closer to membrane renders the membrane distal clone efficient in TDCC. In another series of T-BsAbs against p-Cadherin developed by Root et al., two antibodies that bound to the distal domains of p-Cadherin with high affinities failed to exert any cytotoxic activity (Root, et al., 2016). Thus, evidences available so far consistently suggest that bringing epitopes closer to the cell membrane can be beneficial in inducing more efficient target cell killing. Intuitively, shorter distance between target cells and effector cells could alter the interaction of activating or inhibitory ligand-receptor pairs, or it could directly influence the transport of cytotoxic molecules into the target cells.

Two other important factors pertaining to T-BsAb targets are the expression levels of antigens and the behavior of antigens on cell membrane, i.e., their mobility and distribution pattern. Evidences regarding the effect of antigen expression levels have been inconsistent in the literature. Some studies have shown positive correlation between half maximal effective concentration (EC_{50}) with antigen expression level (e.g., GB1302, hu3F8-BsAb and HER2-BsAb) (Croset, et al., 2014; Lopez-Albaitero, et al., 2017; H. Xu, et al., 2015), while others demonstrated otherwise (e.g., MEDI-565 and AMG-330) (Friedrich, et al., 2014; Oberst, et al., 2014). These results are difficult to reconcile, but they could be attributed to the different assay systems in different laboratories, the number of cell targets examined, the different culture conditions of cell lines, the potency of the specific T-BsAbs, as well as the physical-biochemical properties of the particular antigens studied. Another interesting aspect of antigen that has not been extensively studied is their mobility and distribution pattern on the cell membrane. In a study with human glioblastoma that express both EGFR and CSPG, it was found that EGFR-specific T-BsAb induced higher cytotoxicity than CSPG-specific T-BsAb, although the cell expressed higher CSPG level. Immunofluorescence staining showed that EGFR formed “patchy” staining pattern, whereas CSPG had uniform pattern (Pfosser, Brandl, Salih, Grosse-Hovest, & Jung, 1999). One plausible explanation, albeit speculative, was that antigens that form microclusters on cell surface may have a higher chance of clustering TCR and activate T cells. This would add an interesting dimension to the properties of antigens. However, more studies are obviously required to substantiate such proposals and to untangle the effects of membrane proximity, expression levels, mobility and microclusters of antigens on the potency of T-BsAb. It is also important to note that for T-BsAbs with bivalent antigen binding, extremely high antigen density may in fact generate steric hindrance for T-BsAb binding, whereas too low a density may exceed the maximal distance between the paratopes, as suggested by Plückthun and Pack (Plückthun & Pack, 1997), leading to less effective effector cell activation.

3.2.2. Anti-tumor antigen and anti-CD3 avidity—Under normal conditions, CTLs make transient contacts with target cells that usually result in futile signaling. Higher avidity of T-BsAb may increase the contact time between target cells and effector cells and therefore increase the likelihood of T cell activation. Indeed, by comparing different affinity matured mutants of an anti-p-Cadherin antibody, Root et al. (Root, et al., 2016) demonstrated that

within the same epitope, increasing the affinity from 43.4nM to 3.9nM and 0.2nM also increased the potency by 10 folds and 130 folds, respectively, as measured by EC_{50}. These increases in affinity were mainly the result of decrease in k_{off} by as much as 800 folds, which translated to an increase in interaction $t_{1/2}$ from 1.2min to 16hrs, a timeframe that will increase the efficiency of tumor cytotoxicity. Similarly, data from Reusch et al. showed that in one set of affinity matured TandAb antibodies (T597, T613, T605) derived from the same anti-CD33 clone, increasing the affinity from 9.7nM to 0.7nM decreased the EC_{50} of TandAbs to induce PBMC proliferation from 500pM to 7pM; similar trend was observed for another set of antibodies (T479, T481, T480, T478) fused to a different anti-CD3 clone (Uwe Reusch, et al., 2016). In another affinity maturation experiment using TCR ImmTAC, Liddy et al. (Liddy, et al., 2012) demonstrated that increasing affinities of ImmTAC from 30μM to 0.32nM markedly improved the activation of T cells as measured by IFNγ secretion. However, increasing affinity from 0.32nM to 0.03nM did not seem to further increase T cell activation significantly, suggesting that when a threshold was reached, further decrease in k_{off} (hence increase in $t_{1/2}$) could not increase activation much further, although no detailed kinetic parameters were provided in that study.

To date, 3 reports have compared the effects of changing the avidity of anti-CD3 antibody on cytotoxicity. Bortoletto et al. (Bortoletto, Scotet, Myamoto, D'Oro, & Lanzavecchia, 2002) used mutants of clone TR66 with either increased or decreased affinity to test their ability to activate T cells and exert cytotoxicity. They found that the wildtype version was better than either mutant in T cell activation and cytotoxicity, and that the low affinity mutant was better than the high affinity mutant, suggesting that there exists an optimal kinetics for CD3 binding in order to exert optimal cytotoxicity and that increasing affinity did not always lead to increased function. These interpretations are consistent with observations in classic CTLs, where low affinity interaction of TCR and pMHC is necessary to permit serial TCR triggering (Valitutti, Muller, Cella, Padovan, & Lanzavecchia, 1995). However, it should be noted that the experiments of Bortoletto et al. (Bortoletto, et al., 2002) were done using antibody supernatants from CHO cells and not purified antibodies, potentially weakening their conclusions. Two more recent studies suggested pharmacokinetics and toxicity as important factors in designing high affinity T-BsAbs. In one study, Leong et al. (Leong, et al., 2017) compared the in vitro and in vivo efficacy of 3 highly purified T-BsAbs with CD3 binding affinities of 50nM, 0.5nM and 0.05nM. Variants with lower affinities had EC_{50} that were 4-100 folds higher (less potent) than those with higher affinities, most likely due to the lower activation of CD8(+) T cells. However, higher affinity variants had 2-4 fold faster clearance when injected into mice and they were associated with severe cytokine storm when injected into cynomolgus monkeys. In another study using Xmab13551, an anti-CD38 T-BsAb developed by Xencor, two variants with lower anti-CD3 affinity were compared with the original antibody for CD38(+) cell depletion and cytokine secretion. It was found that in vitro TDCC potency correlated with anti-CD3 affinity. However, when injected in into cynomolgus monkeys, the variant with intermediate anti-CD3 affinity mediated more sustained CD38(+) cell depletion compared to the other two versions; moreover, the it caused less cytokine release than the parental T-BsAb, hence less toxicity (G. L. Moore, et al., 2015). All these suggest that potency, pharmacokinetics and toxicity need to be balanced while manipulating the affinity of the anti-CD3 arm.

3.2.3. Formats of T-BsAbs—More than 32 bispecific formats have been employed for T cell engaging bispecific antibody generation (Table 1 & 2). Currently only the tandem scFv (BiTE) and Triomab trifunctional format have been approved for clinical use, although there are multiple promising formats under active preclinical and clinical development. Different formats differ in molecular size, stability, flexibility, compactness, ease of production, valency of antigen binding, mode of interaction with target cells and effector cells, as well as pharmacokinetics. There is probably no one format suitable for all applications. Which design to use very likely depends on the specific antigen and the specific application, although a few studies have compared side-by-side the potency of different designs with the same antigen binding components.

In an interesting comparison among Fab-Fab (tandem Fab), tandem scFv and full-length IgG (orthogonal Fab) that all target EGFR, it was shown that in a FACS-based cytotoxicity assay, tandem scFv was roughly 10 fold more potent than Fab-Fab format (EC_{50}<10pM vs EC_{50}<100pM), whereas both were more potent than the full-length IgG format (X. Wu, et al., 2015). Full-length IgG is highly flexible in the hinge region, causing the angular distance between the two Fab arms to vary from 20 to 180 degrees, equivalent to be around 10 nm on average (Bongini, et al., 2004; Oda, et al., 2006). Based on the molecular weight, it is possible that the distance between the two paratopes in tandem scFv is closer than those in Fab-Fab and IgG format, hence it is able to bring the effector cells closer to the target cells. This is indeed supported by another study using HER2 specific FynomAb (Wuellner, et al., 2015). In vitro TDCC assay demonstrated that a FynomAb with N-terminal fusion had ~ 8-fold higher potency than the same antibody with C-terminal fusion. In a study that demonstrated a cell-free expression system, anti-EpCAM BiTE and BiTE-Fc (monovalent) were compared with scFv-Fc (two scFv fused to two heavy chains at N-termini); and it was found that BiTE and BiTE-Fc have similar activity in T cell activation and TDCC assay and both were better than scFv-Fc (Y. Xu, et al., 2015), likely for the same reason of paratope distance. However, it should be noted that the relative sizes of the different molecules could also influence T cell interaction with their targets. Contradicting data in the literature also exist. For example, diabody, which is much smaller than $F(ab')_2$ and $scFv_4$-Fc format (4 scFv fused at the N-termini of four antibody chains), had similar potency as the former (Hayashi, et al., 2004) and 500-fold less potent than the latter (Asano, et al., 2007), likely because of the differences in the valency of antigen binding.

Two interesting formats show higher potency than tandem scFv in the context of anti-CD19 T-BsAb. The first one is TandAb (tandem diabody). For example, AFM11 (anti-CD19xanti-CD3 TandAb) when produced in CHO cells was 16-34 fold more potent than tandem scFv derived from blinatumomab (Uwe Reusch, et al., 2015). The second one is DART format (MGD011), which exhibited up to 60-fold higher potency than the tandem scFv format with the same antibody components in TDCC, T cell activation and proliferation, as well as IFNγ secretion (P. A. Moore, et al., 2011). Both molecules have entered clinical trial and it would be interesting to see how they perform in comparison to blinatumomab.

In addition to in vitro potency in TDCC assays, the in vivo efficacy of T-BsAbs is also influenced by their pharmacokinetic profile. Half-life of T-BsAbs in vivo is mainly determined by first-pass renal filtration and antibody recycling. The former is determined by

the size of the molecule – drugs smaller than ~ 60kDa can be rapidly eliminated by glomerular filtration in the kidney. The latter is mediated by the neonatal FcR (FcRn) that can bind to Fc-bearing molecules in the acidic compartment of endosomes and recycle them back to the extracellular space. T-BsAb formats consisting of antibody fragments that are small in size and lack Fc region are quickly eliminated from the body, resulting in shorter half-life and the requirement for continuous infusion. For example, diabody, tandem scFv, $F(ab')_2$ and DART all have short half-lives between 2-8 hours (Cochlovius, Kipriyanov, Stassar, Christ, et al., 2000; Friedrich, et al., 2012; Negri, et al., 1995). TandAb has slightly longer half-lives of 7-22 hours (Kipriyanov, et al., 1999; Uwe Reusch, et al., 2015), whereas Fc containing formats like EM801, MGD011, COVA420, PF-06671008 have half-lives between 96-135 hours (Brack, et al., 2014; L. Liu, et al., 2016; Root, et al., 2016; Seckinger, et al., 2017). Incorporation of the Fc region is beneficial for sustaining the effective concentration of T-BsAbs in serum and tumor, and there seems to be a trend to include Fc in T-BsAbs. Inclusion of Fc creates challenges in generating heterodimers required to form T-BsAbs; fortunately, multiple technologies have now been in place to address this issue.

Higher valency of tumor antigen binding is generally desirable since it usually leads to increased avidity of binding and potency of T-BsAbs, as shown in a functional comparison between tandem scFv and dimeric tandem scFv against GD2 (Ahmed, Cheng, Cheung, & Cheung, 2015). Multiple strategies have been employed to achieve this, e.g., IgG(L)-scFV, triFab-Fc, TandAb, scFv-Fc-scFv, Fynomab and Dock-And-Lock. The effect of valency for CD3 binding is less clear, since there are concerns that bivalent CD3 binding will lead to T cell deletion (Kuhn & Weiner, 2016) and antigen-independent activation. Therefore, many designs have purposely avoided bivalent CD3 binding by creating asymmetric molecules with bivalent tumor antigen binding and monovalent CD3 binding, e.g., triFab-Fc, DART-Fc and BiTE-Fc (monomeric). Some technologies were also developed to create symmetric molecules with monovalent tumor antigen and CD3 binding, e.g., knobs-in-holes, crossMab and Fab-arm exchange. It is unclear at present whether bivalent CD3 binding is problematic, since no antigen-independent activation of T cells was observed in a small number of bivalent-designed T-BsAbs (Lopez-Albaitero, et al., 2017; Uwe Reusch, et al., 2015; Wuellner, et al., 2015; H. Xu, et al., 2015). However, given the protection against organ rejection by the bivalent OKT3 antibody, whether T-BsAb with bivalent designs are more tolerogenic than those with monovalent designs need to be carefully examined.

4. Challenges and perspectives

4.1. Quantity and quality of TILs

With their ability to elicit polyclonal anti-tumor T cell responses, T-BsAbs represent an important alternative strategy to adoptive transfer of engineered T cells (e.g., CAR-T cells). Since they rely on the reprogramming of autologous T cell specificity to kill cancerous cells, the quantity and quality of autologous T cells present will very likely determine the effectiveness of T-BsAbs, as demonstrated ex vivo (Harrington, et al., 2015) and in a small clinical study with catumaxomab (Ströhlein, Lefering, Bulian, & Heiss, 2014). In theory, autologous T cells can be manipulated and expanded ex vivo and infused into patients to be used as effector cells. On this front, it is encouraging to see technological advances in the ex

vivo expansion of autologous T cells for clinical applications (Restifo, Dudley, & Rosenberg, 2012; C. Smith, et al., 2015). Alternatively, in vivo expansion using engineered cytokines (e.g. IL15Ra-IL15) may also be a good option. Future strategies with emphasis in the stratification of patients based on T cell content and activity (Becht, et al., 2016) may be critical for assuring clinical success of T-BsAbs.

4.2. Cytokine release syndrome (CRS)

Common with T cell-based immunotherapies, the injection of T-BsAbs is associated with CRS, characterized by sharp increases in serum levels of inflammatory cytokines such as IL-6, TNFα and IFNγ, as seen with blinatumomab or catumaxomab (Mau-Sørensen, et al., 2015; Teachey, et al., 2013). CRS is thought to be caused by overactivation of immune cells beyond the point where it can no longer be self-contained. In the clinic, such activation may be necessary for the efficacy of the therapeutic agent; but its current management is still suboptimal, leading to life-threatening complications. Although the effects of CRS on tumor microenvironment have not been fully understood, IL-6, TNFα and IFNγ have been linked to tumor growth and/or immune evasion (Abiko, et al., 2015; Fisher, Appenheimer, & Evans, 2014; Landskron, De la Fuente, Thuwajit, Thuwajit, & Hermoso, 2014).

Since cytokine release is intimately linked to T cell activation, the same factors that affect the potency of T-BsAb will likely also affect cytokine releases. For example, increasing the affinity of ImmTAC molecule to pMHC increases IFN secretion (Liddy, et al., 2012); similarly, increasing the affinity of anti-CD3 in the context of anti-CLL1 T-BsAb resulted in life-threatening cytokine release in cynomolgus monkeys (Leong, et al., 2017). It should be noted that T cell activation is a loose term that entails different T cell behaviors, including but not limited to upregulation of surface activation markers, release of cytokines, and release of cytotoxic molecules. It is not entirely clear at present if these events can be individually manipulated by changing the T-BsAbs. However, in an interesting study using an anti-PSMA T-BsAb, Hernandez-Hoyos et al. demonstrated that it was possible to generate potent TDCC in vitro while reducing cytokine secretion via the use of scFv-Fc-scFv T-BsAb format (Hernandez-Hoyos, et al., 2016), suggesting that tumor cytotoxicity and cytokine storm may be separable events or an optimal balance between potency and toxicity can be achieved by changing T-BsAb design. A better understanding of how the molecular formats and other T-BsAb properties (e.g., affinities) influence these events will undoubtedly be instrumental in the future design of better T-BsAb candidates to alleviate CRS and avoid cytokine-induced enhancement of tumor growth.

4.3. Specific tumor target antigens

The third challenge faced by T-BsAb therapeutics, or rather monoclonal antibody therapeutics in general, is the identification of therapeutic targets that are specific enough to discriminate between cancer cells and normal cells. Most of the therapeutic targets for antibody drugs under development are differentiation markers that are also expressed in normal cells, albeit at a lower level or with a more restricted pattern. This narrows the therapeutic window and poses a great challenge to the design and development of T-BsAbs. Most of the drugs developed to date represent compromises between clinical efficacy and toxicity. An “ideal target” that can clearly demarcate normal versus diseased cells has yet to

be discovered. Intracellular oncoproteins, which comprise most of the cancer “drivers” and some of which can be presented by pMHC, seem to be the closest we can ascertain as an “ideal target”. However, targeting these antigens requires isolation of TCR or TCR-like antibodies, with substantial issues of cross-reactivity which could compromise organ or tissue selectivity. With the ability to produce more and more high quality “omics” data and their wide accessibility, future target discovery and testing will require integration, plus sophisticated analysis and interpretation of big data. The caveat of this endeavor is that targets may not be of sufficient density, and will likely require extensive testing before specificity can be proven. To create safe and effective clinical T-BsAb for such targets will take time.

4.4. T cell homing, activation and survival

To date, no data on T cell homing are available in the clinic for targeting solid tumors using intravenous T-BsAbs. The ideal T-BsAb should drive T cells into solid tumors, activate TILs to proliferate, and carry out the anti-tumor function despite the presence of immune checkpoints in the tumor stroma. Successful T-BsAb therapy requires the efficient implementation of each of these steps. However, multiple obstacles exist (Figure 2).

First of all, T cells, whether they are already residing in the tumors (TILs) or are driven by T-BsAb to infiltrate tumors, are the ingredients of “inflamed tumors”, a prerequisite for response to T cell-based therapies. Preclinical studies have consistently shown that the presence of TILs correlates with therapeutic efficacy of immune checkpoint inhibitors (Gajewski, et al., 2013; Gooden, et al., 2011; Spranger, 2016); one might expect it to also predict tumor response to T-BsAbs. However, T cell numbers may be low due to depletion by prior chemotherapy or cachexia. The hostile microenvironment of tumor, which is hypoxic, acidic and immunosuppressive, could also contribute to the suppression and further deletion of TILs (Bellone & Calcinotto, 2013). Furthermore, exhaustion may also be due to the repeated over-stimulation of TILs by T-BsAbs. In an in vitro serial TDCC assay, Osada et al. showed that the cytotoxicity of T cells induced by anti-CEA BiTE was impaired when the same T cells were transferred from the first culture and applied to a second culture of target cells (Osada, et al., 2015). In a syngeneic mouse model, T-BsAb could cause apoptosis of TILs by reactivation induced cell death (Hettich, Lahoti, Prasad, & Niedermann, 2016). Thus, how to maintain and expand a functional population of cytotoxic T cells is a pressing question faced by T-BsAb therapy. It may be unrealistic to expect T-BsAb to provide all the signals for these purposes inside the tumor stroma. In this regard, cytokines such as IL15 are viable candidates to aid the survival and expansion of T cells (Huarte, et al., 2009; Rettinger, et al., 2012).

Second, T cells recruited by T-BsAbs need to overcome high interstitial pressure and a molecular network that “discourages” their entry. Tumor vasculatures are highly disorganized; and tumors secreted factors like VEGFα and FGFs can cause tumor endothelial cells to become “anergic”, i.e., losing adhesion molecules like ICAM-1/2, VCAM-1 and CD34, which are important for T cell transmigration (Bellone & Calcinotto, 2013). Moreover, tumor cells can secrete chemokines that attract immunosuppressive cells and repel CTLs (Oelkrug & Ramage, 2014). How and whether T-BsAbs can help overcome

these limitations remain to be seen. However, in xenograft models, some T-BsAb formats (e.g. IgG(L)-scFv) can drive T cells from the blood into solid tumors and effect tumor ablation, despite the upregulation of PD-L1 in the tumor stroma (Lopez-Albaitero, et al., 2017; H. Xu, et al., 2015). It is noteworthy also that inflammatory cytokines secreted by T-BsAb activated T cells, e.g., TNFα, can apparently overcome some of these barriers, as demonstrated by an NGR-TNF molecule that specifically targeted tumor vasculature through NGR peptide and that was able to increase infiltration of CD8(+) T cells (Calcinotto, et al., 2012). This molecule is currently in late clinical development. It would be interesting to test the combination of vasoactive cytokines with T-BsAbs for solid tumor immunotherapy. Apart from cytokines, which usually have pleiotropic effects, CTL-attracting chemokines (e.g., CXCL9, CXCL10, CCL3) may be another viable alternatives, e.g., by incorporating a chemokine component in T-BsAb designs. The challenge with such designs will be the maintenance of a chemokine concentration gradient between blood and tumor, which is essential for chemokines to recruit T cells (Siddiqui, Erreni, van Brakel, Debets, & Allavena, 2016); extensive testing and optimization will be required both in vitro and in vivo.

Another obstacle for T cell based immunotherapy is the prevalence of immunosuppressive molecules in the tumor microenvironment, both cell surface bound and secreted. Tumor endothelial cells, immunosuppressive myeloid cells, and tumor cells can all express immunosuppressive molecules like PD-L1 and PD-L2 (Motz & Coukos, 2011; W. Zou, Wolchok, & Chen, 2016). In this regard, the ability of T cells to survive and not become exhausted, while being driven by T-BsAb into the tumor, should benefit from the explosive developments in the field of ICIs. In preclinical models, Junttila et al. demonstrated in a HER2(+) CT26 subcutaneous model that combining anti-PD-L1 antibody and anti-HER2 T-BsAb induced a stronger and a more durable anti-tumor response, when compared to T-BsAb alone (Junttila, et al., 2014). Similarly, combination of anti-PD-1 or anti-PD-L1 antibodies with CD20-TDB also showed greater anti-tumor effects than single-agents alone (Liping Laura Sun, et al., 2016). In clinical settings, screening of co-signaling molecules on B-ALL blasts from blinatumomab treated patients identified PD-L1 as the marker that was significantly upregulated in non-responders. Moreover, combination of blinatumomab and pembrolizumab induced responses in a previously nonresponding 12-year-old patient (Feucht, et al., 2016). Clinical trials of similar combination therapies for solid tumors are currently underway, e.g., RO6958688 (anti-CEA T-BsAb) is in a phase 1b study in combination with atezolizumab to treat CEA-positive tumors.

One interesting proposal to enhance T cell homing, infiltration and survival is to combine T-BsAb with low-dose chemotherapy. Although high dose of chemotherapy is immunosuppressive, low dose of selected chemotherapy can modify subsets of T cells to the host's advantage. In a murine mesothelioma model, the addition of cisplatin to anti-CTLA4 enhanced infiltration of TILs accompanied by higher anti-tumor efficacy (L. Wu, Yun, Tagawa, Rey-McIntyre, & de Perrot, 2012). Fan et al. also showed that low-dose cytosine arabinoside increased the expression of CD80 and CD86 on B-ALL patient-derived samples, sensitizing them to anti-CD19 T-BsAb killing (Fan, et al., 2015). In summary, the success of T-BsAbs will likely require a combination of strategies and modalities to enhance T cell homing, activation and proliferation, as well as derepression in the tumor microenvironment.

Some of these can be integrated into the design of T-BsAbs to include more specificity, whereas others may be better achieved with a cocktail of drugs/biologics.

5. Conclusions

This is an exciting decade for antibody drug development. Since the discovery of monoclonal antibody technology, over the last 30 years, advances in protein engineering and manufacture, coupled with better understanding of cancer biology and immunology, have enabled better design and faster clinical translation of novel immunotherapeutics to address unmet medical needs. Future progress in the fight against cancer will likely require the integration of multiple ttreatment strategies, and antibodies will play a pivotal role. In addition to normal IgG antibodies, immune checkpoint inhibitors, antibody drug conjugates, radioimmunoconjugates, and CAR-T-cells, T-BsAbs provide another exciting and potent class of antibody-based immunotherapeutics, whose potential has yet to be fully realized. Although currently only two T-BsAb antibodies have been approved for clinical use, with more than 60 bispecific designs and some promising candidates, we are optimistic that more approvals will follow in the near future.

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Abbrevation

CRS	cytokine release syndrome
CTL	cytotoxic T lymphocyte
EC_{50}	half maximal effective concentration
Fc	fragment crystallizable
ICI	immune checkpoint inhibitor
pMHC	peptide-major histocompatibility complex
scFv	single chain variable fragment
TandAb	tandem diabody
T-BsAb	T cell engaging bispecific antibody
TCR	T cell receptor
TDCC	T cell dependent cellular cytotoxicity
TIL	tumor infiltrating lymphocyte

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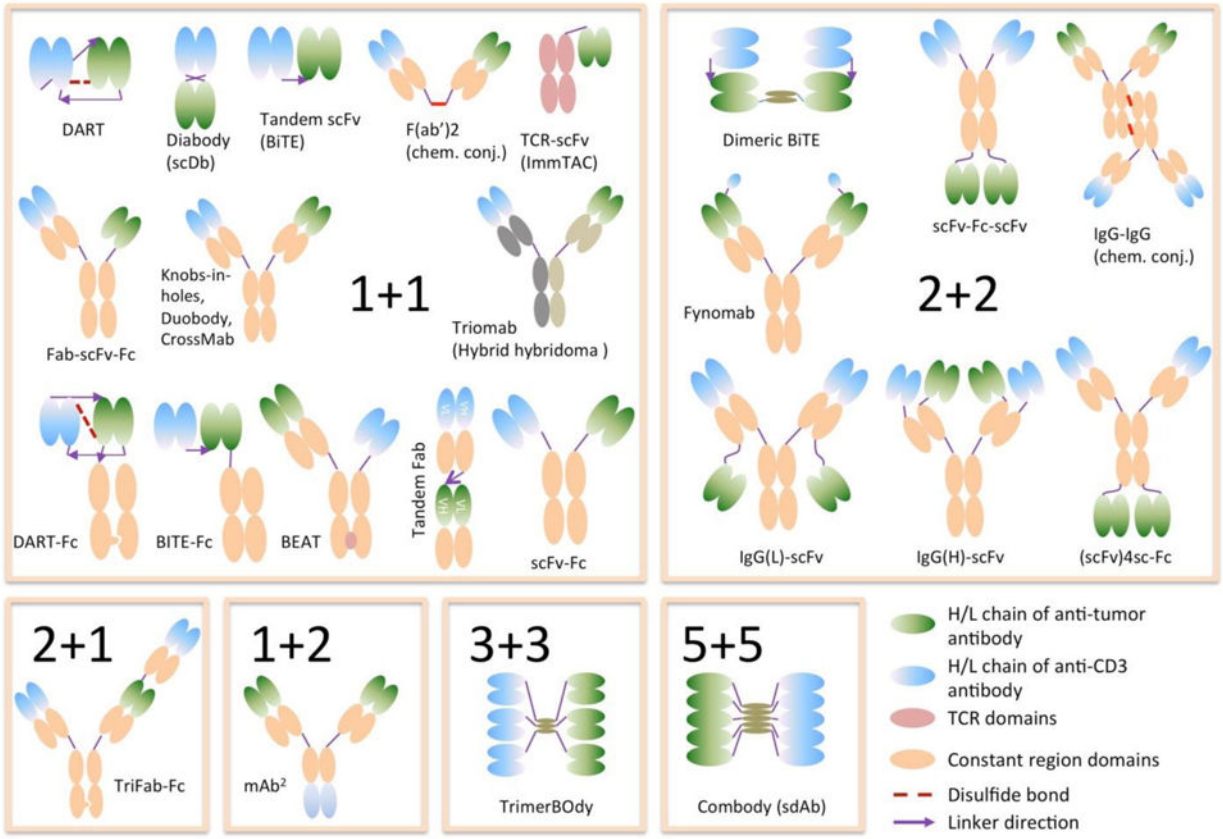

Figure 1. Different formats of T-BsAbs

The different molecular designs are grouped by the valency of binding to tumor antigen (first number) and the valency of binding to CD3 (second number). For example, 2+1 denotes bivalent tumor antigen binding and monovalent CD3 binding.

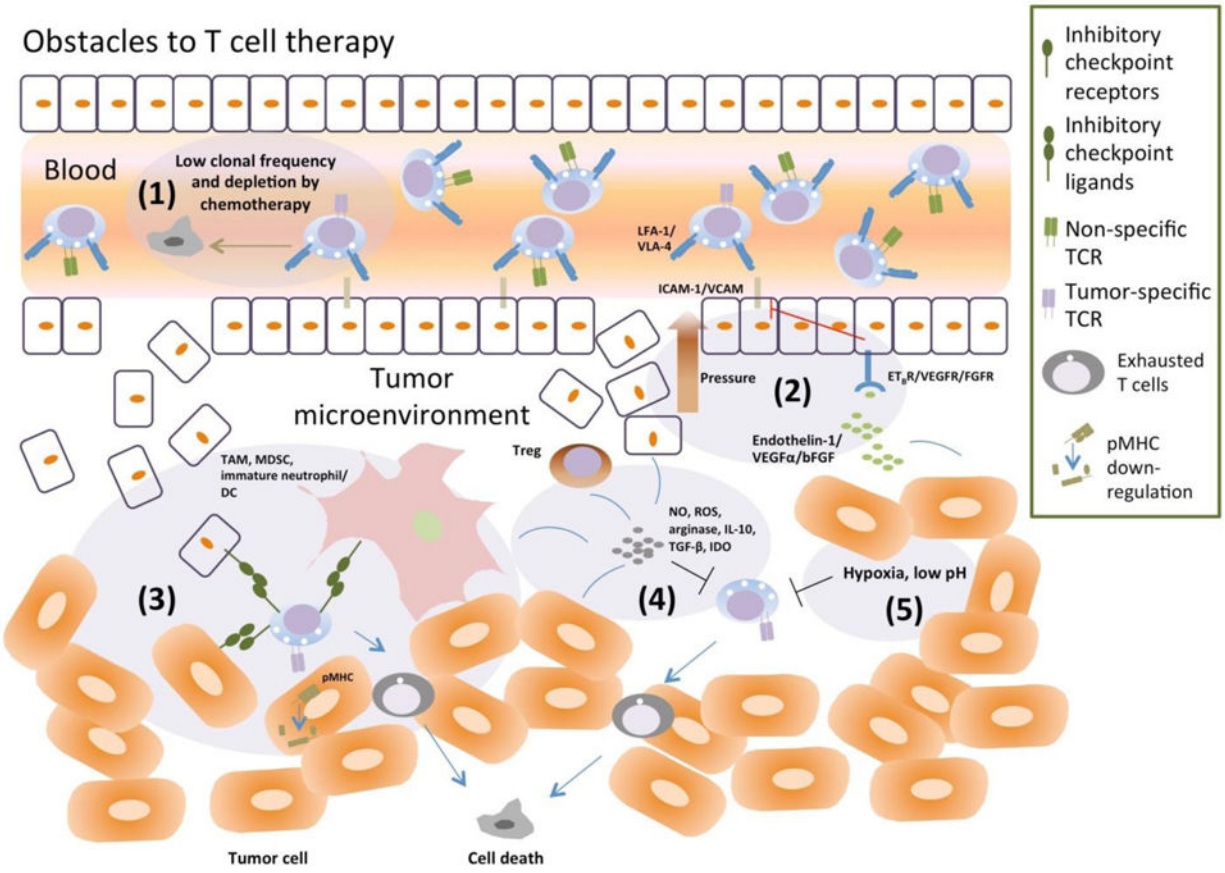

Figure 2. Hurdles for T cell-mediated tumor surveillance

Insufficient tumor infiltrating lymphocytes (TILs) (both antigen-specific and antigen-nonspecific) can be caused by: (1) low clonal frequency of tumor specific T cells and depletion of lymphocytes by chemotherapy; (2) denial of T cell entry due to increase in interstitial pressure (abnormal angiogenesis and irregular endothelium) and down-regulation of adhesion molecules on endothelial cells (“anergic” EC), both controlled by soluble factors (e.g. endothelin-1, VEGFα and bFGF) secreted by tumor cells and other immunosuppressive cells present in the tumor microenvironment (TME). Mechanisms used by tumor cells to evade T cell killing mainly consist of: (3) downregulation of MHC and the cognate T-cell receptor (TCR) target (peptide-MHC) on tumor cells and suppression by inhibitory immune checkpoint receptor-ligand interactions (e.g. PD1 with PD-L1/PD-L2, CTLA4 with CD80/CD86); (4) anergy mediated by secreted immunosuppressive molecules (e.g., NO, ROS, arginase, IL-10, TGFβ, IDO); (5) alteration by tumor cells of the metabolic environment making it hypoxic and acidic, which can be detrimental to T cell function. Processes (2)-(5) can be executed by the different cellular components in the TME, such as tumor cells, endothelial cells, regulatory T cells (Treg), tumor associated macrophage (TAM), myeloid derived suppressor cells (MDSC), immature neutrophil and immature DC

Table 1

T-BsAb in clinical development*

Name	Clinical Phase[1]	Tumor Antigen	αCD3 clone used[2]	Formats	References
AMG 420 (a.k.a.duvortuxizumab, BI 836909)	I (2015/NCT02514239)	BCMA	n.a.	BiTE	(Hipp, et al., 2017)
JNJ-63709178	I (2016/NCT02715011)	CD123	n.a.	hIgG	(Gaudet, et al., 2016)
MGD006	I (2014/NCT02152956)	CD123	proprietary	DART	(Chichili, et al., 2015; L. Huang & Johnson, 2014)
XmAb14045	I (2016/NCT02730312)	CD123	n.a.	Fab-scFv-Fc	(Chu, Pong, et al., 2014)
AFM11	I (2014/NCT02106091)	CD19	UCHT1 (h)	TandAb	(Uwe Reusch, et al., 2015)
MGD011 (a.k.a. JNJ-64052781)	I (2016/NCT02743546)	CD19	XR32 (h)	DART-Fc	(L. Liu, et al., 2016)
MT103 (Blinatumomab)	Approved	CD19	L2K	BiTE	(Dreier, et al., 2003; Dreier, et al., 2002; Löffler, et al., 2000; Mølhøj, et al., 2007)
Bi20 (FBTA05)	I/II (2010/NCT01138579)	CD20	26II6 (r)	m/rIgG	(Stanglmaier, et al., 2008)
CD20-TDB (a.k.a. BTCT4465A, RG7828)	I (2015/NCT02500407)	CD20	UCHT1 (h)	hIgG	(Liping L. Sun, et al., 2015)
REGN1979	I (2014/NCT02290951)	CD20	n.a.	hIgG	(E. J. Smith, et al., 2015) From VelocImmune mice
AMG-330	I (2015/NCT02520427)	CD33	n.a.	BiTE	(Friedrich, et al., 2014; Harrington, et al., 2015; Laszlo, Gudgeon, Harrington, & Walter, 2015)
CEA TCB (RG7802, RO6958688)	I (NCT02324257 and NCT02650713)	CEA	proprietary	TriFab-Fc	(Bacac, et al., 2016)
MEDI-565 (a.k.a. AMG-211)	I (2011/NCT01284231)	CEA	L2K (de)	BiTE	(Oberst, et al., 2014)
MCLA-117	I (2017/NCT03038230)	CLEC12A, a.k.a. CLL-1	proprietary	hIgG	(Bakker, VAN LOO, & Logtenberg, 2014; Van Loo, Doornbos, Dolstra, Shamsili, & Bakker, 2015)
AMG110 (a.k.a. MT110, Solitomab)	I (2008/NCT00635596)	EpCAM	L2K (de)	BiTE	(Brischwein, et al., 2006; Herrmann, et al., 2010)
Catumaxomab	Approved	EpCAM	26II6 (r)	m/rIgG	(Chelius, et al., 2010; Ruf, et al., 2004; Zeidler, et al., 1999)
MGD007	I (2014/NCT02248805)	GPA33	n.a.	DART-Fc	(P. A. Moore, et al., 2014)
ERY 974	I (2016/NCT02748837)	GPC3	n.a.	hIgG	(Ishiguro, et al., 2016)
Ertumaxomab	II (2007/NCT00522457)	Her2	26II6 (r)	m/rIgG	
GBR1302	I (2016/NCT02829372)	Her2	n.a.	BEAT	(Croset, et al., 2014)
IMCgp100	Ib/II (2015/NCT02535078)	HLA-A2/gp100	n.a.	TCR-αCD3	(Liddy, et al., 2012)

Name	Clinical Phase[1]	Tumor Antigen	αCD3 clone used[2]	Formats	References
PF-06671008	I (2016/NCT02659631)	p-cadherin	XR32 (h)	DART-Fc	(Root, et al., 2016)
BAY2010112 (AMG212, Pasotuxizumab)	I (2012/NCT01723475)	PSMA	proprietary	BiTE	(Friedrich, et al., 2012; WHO, 2014)
MOR209/ES414	I (2014/NCT02262910)	PSMA	n.a.	scFv-Fc-scFv	(Hernandez-Hoyos, et al., 2016)

*This table excludes trials using pre-arm ATC.

[1]Clinical trial stage shows the most advanced clinical phases for the molecule to date. The year of the trial is based on the date published on clinicaltrials.gov.

[2]n.a. denotes clones whose information is not disclosed in the literature; proprietary denotes clones whose information is available in the patent issued or patent pending, as cited in the references; (h):humanized; (r):rat; (de):deimmunized.

Table 2

Past and existing T-BsAbs under preclinical development

Name[1]	Target Antigen	Format/Year[2]	αCD3 clone used[3]	Reference
A300E-BiTE	ADAM17	BiTE/2012	L2K	(Yamamoto, et al., 2012)
BiFab-BCMA	BCMA	Chem. Conj via unnatural aa/2015	UCHT1	(Ramadoss, et al., 2015)
EM801	BCMA	TriFab-Fc/2017	n.a.	(Seckinger, et al., 2017)
CD10xCD3	CD10	F(ab')2 by Chem. Conj/1991	OKT3	(Oshimi, et al., 1991)
CD123xCD3	CD123	scFv-Fc-scFv/2012	UCHT1	(Kuo, Wong, & Liu, 2012)
Xmab14045	CD123	Fab-scFv-Fc/2014	n.a.	(Chu, Pong, et al., 2014)
CD133xCD3	CD133	Chem. conj+pre-armed ATC/2013	OKT3	(J. Huang, et al., 2013)
MS133	CD133	Fab-scFv-Fc/2015	OKT3 (h)	(Zhao, 2015)
STL001	CD138	BiTE-Fc/2015	(Kufer, Lutterbuse, Kohleisen, Zeman, & Bauerle, 2009)	(J. Zou, et al., 2015)
(19)-3s	CD19	Dock-and-lock/2014	OKT3	(D. L. Rossi, Rossi, Cardillo, Goldenberg, & Chang, 2014)
bscCD19xCD3	CD19	BiTE/2000	TR66	(Löffler, et al., 2000)
CD19xCD3	CD19	Hybrid hybridoma/1998	OKT3	(Daniel, et al., 1998)
CD19xCD3	CD19	Tandab/1999	OKT3	(Cochlovius, Kipriyanov, Stassar, Schuhmacher, et al., 2000; Kipriyanov, et al., 1999)
CD19xCD3	CD19	Diabody/2000	OKT3	(Cochlovius, Kipriyanov, Stassar, Christ, et al., 2000)
CD19xCD3	CD19	DART/2011	TR66	(P. A. Moore, et al., 2011)
CD19xTCR	CD19	DART/2011	hBMA031	(P. A. Moore, et al., 2011)
HD37xT5.16	CD19	Hybrid hybridoma/2007	Anti-CD5	(Tita-Nwa, et al., 2007)
(20)-3s	CD20	Dock-and-lock/2014	n.a.	(D. L. Rossi, et al., 2014)
BIS20X3	CD20	F(ab')2 by Chem. Conj/2004	37-6673	(Stel, et al., 2004)
CD20xCD3	CD20	Diabody/2002	HIT3a	(Xiong, et al., 2002)
CD20xCD3	CD20	Chem. conj+pre-armed ATC/2005	OKT3	(Gall, Davol, Grabert, Deaver, & Lum, 2005)
CD20XCD3	CD20	IgG(H)-scFv/2016	n.a.	(Lu, et al., 2016)
(22)-3s	CD22	Dock-and-lock/2014		(D. L. Rossi, et al., 2014; E. A. Rossi, Rossi, Cardillo,

Name[1]	Target Antigen	Format/Year[2]	αCD3 clone used[3]	Reference
				Chang, & Goldenberg, 2014)
CD22XCD3-RicinA	CD22	Hybrid hybridoma/1994	64.1 (mIgG2a)	(Shen, Li, & Vitetta, 1994)
CD30xCD3	CD30	Hybrid hybridoma/1993	OKT3	(Pohl, et al., 1993; Renner & Pfreundschuh, 1995)
AMV564	CD33	TandAb/2016	n.a.	(Uwe Reusch, et al., 2016)
CD33xCD3	CD33	BiTE/2011	n.a.	(Stamova, et al., 2011)
CD33xCD3	CD33	Pre-targeting/2014	MT-301	(Arndt, et al., 2014)
Xmab13551	CD38	Fab-scFv-Fc/2014	n.a.	(Chu, Miranda, et al., 2014)
aCEAxaCD3	CEA	Diabody/2003	OKT3	(Blanco, Holliger, Vile, & Álvarez-Vallina, 2003)
CEAxCD3	CEA	BiTE/2015	L2K (de)	(Osada, et al., 2015)
MF23B/OKT3	CEA	Diabody/1999	OKT3	(Holliger, et al., 1999)
Claudin6XCD3	Claudin6	BiTE/2016	TR66	(Stadler, et al., 2016)
CCL1xCD3	CLL-1	hIgG/2017	n.a.	(Leong, et al., 2017)
CMVBi	CMV	Chem. conj+pre-armed ATC/2012	OKT3 (m)	(Lum, et al., 2012)
BiAb(OKT3x cetuximab)	EGFR	Chem. conj+pre-armed ATC/2006	OKT3	(Ursula Reusch, et al., 2006)
biMAbM26. 1	EGFR	Hybrid hybridoma/1993	289.1 (mIgG2a)	(Ferrini, et al., 1993; Negri, et al., 1995)
CD3xEGFR	EGFR	Orthogonal Fab	n.a.	(Lewis, et al., 2014)
CD3xEGFR	EGFR	Tandem Fab, BiTE, IgG	n.a.	(X. Wu, et al., 2015)
hEx3	EGFR	Diabody, $scFv_4$-Ig and scDb-Fc/ 2004	OKT3	(Asano, et al., 2014; Asano, et al., 2007; Hayashi, et al., 2004; Watanabe, 2011)
M2xEGFR	EGFR	F(ab')2/1999	Anti-CD2	(Wild, Strittmatter, Matzku, Schraven, & Meuer, 1999)
EGFRvIIIxCD3	EGFRvIII	BiTE/2013	OKT3	(Choi, et al., 2013)
1H8/CD3	EpCAM	BiTE/2014	(Dorken, et al., 2006)	(Zhang, et al., 2014)
BiTE-KIH	EpCAM	BiTE, BiTE-Fc/2015	diL2K	(Y. Xu, et al., 2015)
E3Bi	EpCAM	BiTE/2004	n.a.	(Ren-Heidenreich, Davol, Kouttab, Elfenbein, & Lum, 2004)
EpCAMxCD3	EpCAM	BiTE/1997	TR66	(Mack, Gruber, Schmidt, Riethmüller, & Kufer, 1997)
HEA125XOKT3	EpCAM	Hybrid hybridoma/2009	OKT3	(Salnikov, et al., 2009)

Name[1]	Target Antigen	Format/Year[2]	αCD3 clone used[3]	Reference
EphA10xCD3	EphA10	BiTE/2015	OKT3	(Taki, et al., 2015)
FcRH5xCD3	FcRH5	hIgG/2017	n.a.	(Li, et al., 2017)
TR66XMOv18	Folate Receptor	F(ab')2 by Chem. Conj/1991	TR66	(Mezzanzanica, et al., 1991)
G250XCD3	G250	Chimeric IgG/1996	4B5	(Luiten, Coney, Fleuren, Warnaar, & Litvinov, 1996)
hu3F8-BsAb	GD2	IgG(L)-scFv/2015	OKT3 (h)	(Cheng, Ahmed, Xu, & Cheung, 2015; H. Xu, et al., 2015)
hCD4IgGxCD3	gp120	Fab-Fc+hCD4-Fc/1994	UCHT1	(Chamow, et al., 1994)
Janusins	gp120	scFv-Fc+hCD4-Fc/1991	n.a.	(Traunecker, Lanzavecchia, & Karjalainen, 1991)
VRC07xCD3	gp120	Tandem FAB (VRC07)-scFv/2015	n.a.	(Pegu, et al., 2015; Petrovas, et al., 2017)
HIVxCD3	gp120/gp41	DART/2015	XR32	(Sung, et al., 2015)
CD3xAntag2	GRPR	Chem. Conj/2006	OKT3	(Zhou, et al., 2006)
COVA420	HER2	FynomAb/2014	OKT3 (h)	(Brack, et al., 2014; Wuellner, et al., 2015)
FcabCD3	HER2	mAb[2]/2010	(Hofmeister, et al., 2005)	(Wang, et al., 2013; Wozniak-Knopp, et al., 2010)
HER2-BsAb	HER2	IgG(L)-scFv/2017	OKT3 (h)	(Lopez-Albaitero, et al., 2017)
HER2xCD3	HER2	F(ab')2 by Chem. Conj/1992	UCHT1 (h)	(M R Shalaby, 1992)
HER2xCD3	HER2	F(ab')2 by Chem. Conj/1993	OKT3	(Tsukamoto, et al., 1993)
HER2xCD3	HER2	Chem. conj+pre-armed ATC/2001	OKT3	(Sen, et al., 2001)
HER2xCD3	HER2	F(ab')2 by Chem. Conj/2002	UCHT1 (h)	(Scheffold, Kornacker, Scheffold, Contag, & Negrin, 2002)
HER2xCD3	HER2	F(ab')2 by by unnatural aa/2012	UCHT1	(Kim, et al., 2012)
HER2xCD3	HER2	Chem. conj+pre-armed ATC/2014	OKT3	(Han, 2014)
Her2xCD3	HER2	Universal adaptor/2014	n.a.	(H. Y. Liu, Zrazhevskiy, & Gao, 2014)
HER2xCD3	HER2	Tetra-IgG, tri-IgG, Tri-Fab, BiFab by unnatural amino acid/2015	UCHT1	(Cao, et al., 2015)
mAb-Fv	HER2	IgG(H)-scFv/2011	OKT3 (h)	(G. L. Moore, et al., 2011)
HER2xCD3/CA-125xCD3	HER2/CA-125	Chem. Conj/2006	OKT3	(Chan, et al., 2006)
pMHCxCD3	HLA-A2/AFP15 8-166	BiTE/2017	n.a.	(H. Liu, et al., 2017)
pMHCxCD3	HLA-A2/NY-ESO-1, LAGE-1, gp100, MAGE-A3, Melan-A	ImmTAC/2012	n.a.	(Liddy, et al., 2012; McCormack, et al., 2013)

Name[1]	Target Antigen	Format/Year[2]	αCD3 clone used[3]	Reference
pMHCxCD3	HLA-A2/WT1	BiTE/2015	n.a.	(Dao, et al., 2015)
pMHCxCD3	HLA-A2-MART-1	Combody (sdAb)/2010	n.a.	(Zhu, et al., 2010)
(x)-3s	HLA-DR, CEAC AM5, CEA CAM6, Trop-2	Dock-and-lock/2014	n.a.	(D. L. Rossi, et al., 2014; E. A. Rossi, et al., 2014)
LamininxCD3	Laminin	Trimerbody/2013	OKT3	(Blanco-Toribio, et al., 2013)
MCSPxCD3	MCSP	BiTE/2011	n.a.	(Torisu-Itakura, et al., 2011)
	MCSP (CSPG4)	BiTE/2010	L2K	(Bluemel, et al., 2010)
SEA D227A-Mx3	MUC-1	SA-fused to diabody/2002	OKT3	(Takemura, et al., 2002)
5.2-CD3	PfMSP-1_{19}	BiTE/2003	OKT3 (m)	(Yoshida, et al., 2003)
PgpxCD3	pgp	dsDb (disulfide diabody)/2004	HIT3a	(Gao, et al., 2004; J. Liu, et al., 2009)
PSCAxCD3	PSCA	scDiabody, BiTE/2011	MT-301	(Feldmann, et al., 2012; Feldmann, et al., 2011)
PSMA	PSMA	Diabody/2008	n.a.	(Bühler, et al., 2008)
Xmab18087	SSTR2	Fab-scFv-Fc/2017	n.a.	(Lee, et al., 2017)
TenascinxCD3	Tenascin	hybrid hybridoma/1995	CBT3G	(Bonino, et al., 1995)
Fab-sec conjugate	α4β7, folate receptor	Fab-sec conjugate/2012	v9 (h)	(Cui, Thomas, Burke, & Rader, 2012)

[1] T-BsAbs that did not have a specific name from the reference were named as antigenxCD3.

[2] year after "/" is the year of publication.

[3] n.a. denotes clones whose information is not available.