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REVIEW

Bispecific antibodies in cancer immunotherapy

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ABSTRACT
Cancer immunotherapy has recently generated much excitement after the continuing success of the immunomodulating anti-CTLA-4 and anti-PD-1 antibodies against various types of cancers. Aside from these immunomodulating antibodies, bispecific antibodies, chimeric antigen receptor T cells, and other technologies are being actively studied. Among the various approaches to cancer immunotherapy, 2 bispecific antibodies are currently approved for patient care. Many more bispecific antibodies are now in various phases of clinical development and will become the next generation of antibody-based therapies. Further understanding of immunology and advances in protein engineering will help to generate a greater variety of bispecific antibodies to fight cancer. Here, we focus on bispecific antibodies that recruit immune cells to engage and kill tumor cells.

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Introduction

Cancer is the second leading cause of mortality in developed countries, and the third leading cause of death in developing countries. After several decades of intensive research and development, the survival rate of cancer patients has been dramatically improved for most cancers. For example, chronic myeloid leukemia (CML), which used to be a fatal disease caused by the fusion oncogene BCR-ABL, is now a manageable condition due to the introduction of tyrosine kinase inhibitors.[1] For many other cancers, however, there is still a lack of effective treatments, especially treatments that can result in long-term cancer-free survival.

Cancer immunotherapy was proposed decades ago but has only recently been realized as a promising approach to revolutionize cancer treatment. Cancer immunotherapy, which harnesses the body's immune system to fight cancer, was named "2013's Breakthrough of the Year" by *Science*.[2] The promise of cancer immunotherapy comes from the success of immune checkpoint antibodies, such as the anti-CTLA-4 monoclonal antibody (ipilimumab) and 2 anti-PD-1 monoclonal antibodies (nivolumab and pembrolizumab), which are efficacious against a variety of advanced solid tumors including melanoma, non-small cell lung carcinoma, and renal carcinoma.[3] These studies showed that modulation of the immune system is a viable way to combat cancer. In contrast to traditional chemotherapy and targeted therapy, which mainly focus on cancer cells, cancer immunotherapy helps activate the immune system in patients to recognize and fight cancer cells.[4]

In addition to immunomodulating antibodies, bispecific antibodies are another promising strategy to combat cancer by directly engaging immune cells in the fight against tumor cells. A bispecific antibody is based on a conventional monoclonal antibody. However, while a conventional antibody only binds one specific target, a bispecific antibody can recognize and bind 2 different antigens simultaneously. In light of this advantage, bispecific antibodies can be designed to inhibit 2 cell surface receptors or ligands. This effect, admittedly, can also be achieved with a monoclonal antibody strategy by combining 2 or even multiple antibodies together; however, a bispecific strategy offers an opportunity to reduce cost in terms of development, production clinical trials, and regulatory reviews, compared to the single antibody-based agents developed in combination therapies.[5,6] Another advantage of a bispecific antibody is that it can redirect immune effector cells to the proximity of tumor cells, which is not achievable with a combination monoclonal antibody strategy. Currently, 2 bispecific antibodies, catumaxomab (anti-EpCAM and anti-CD3) and blinatumomab (anti-CD19 and anti-CD3), have been approved for patient care and have made a substantial impact on both research and the development of biologics.[7-9]

Many more diverse formats for bispecific antibodies are now in transit from the bench to bedside (Table 1). This review is focused on bispecific antibodies that recruit immune cells for cancer therapy, which was first demonstrated *in vitro* 30 y ago.[10] Most of the bispecific antibodies are specific to CD3 to recruit T cells to tumor cells, which in turn are targeted by a variety of tumor antigen-specific antibodies. The recruited T cells then exert potent cytotoxicity toward the tumor cells. In addition to T cells, natural killer (NK) cells and dendritic cells (DCs) have also been targeted by bispecific antibodies (Fig. 1).

Strategies to generate a bispecific antibody

Bispecific antibodies can be generated in multiple ways. Chemical conjugation of 2 different purified monoclonal antibodies

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Table 1. Bispecific antibodies in clinical development.

Name	Format	MW(kDa)	Targets	Immune cells engaged	Indication	Development status
Catumaxomab	TrioMab	150	EPCAM + CD3	T cell	Malignant ascites	Approved
					Ovary cancer	Phase II
					Gastric cancer	Phase II
					Epithelial cancer	Phase I
Lymphomum (FBTA05)	TrioMab	150	CD20 + CD3	T cell	BCL	Phase I
Ertumaxomab	TrioMab	150	HER2 + CD3	T cell	Metastatic breast cancer	Phase I
Blinatumomab (AMG 103)	BiTE	50	CD19 + CD3	T cell	B cell ALL	approved
					ALL relapsed refractory	Phase II
					DLBCL	Phase II
					NHL	Phase I
Solitomab (AMG 110)	BiTE	50	EPCAM + CD3	T cell	Colorectal cancer	Phase I
					Lung and gastrointestinal cancer	Phase I
AMG 211 (MEDI-565)	BiTE	50	CEA + CD3	T cell	Gastrointestinal cancers	Phase I
MT 112 (BAY2010112)	BiTE	50	PSMA + CD3	T cell	Prostate cancer	Phase I
MGD006	DART	50	CD123 + CD3	T cell	AML	Phase I
MGD007	DART + Fc	100	gpA33 + CD3	T cell	Colorectal cancer	Phase I
AFM11	TandAb	100	CD19 + CD3	T cell	NonHodgkin's lymphoma	Phase I
AFM13	TandAb	100	CD30 + CD16	NK cell	Hodgkin's lymphoma	Phase I
IMCgp100	ImmTAC	75	Gp100 + TCR	T cell	Malignant melanoma	Phase II
					melanoma	Phase I
rM28	Tandem scFv	50	MAPG + CD28	T cell	Metastatic melanoma	Phase II

was used to make bispecific antibodies by oxidative recombination more than 50 y ago[11] and yielded 2 bispecific antibodies that are currently in clinical development.[12,13] More recently, genetic engineering has been used with increasing frequency to create various types of bispecific antibodies. During the past 2 decades, more than 50 formats have been proposed, and some are in different clinical trials phases for the treatment of cancer or autoimmune diseases.[4-6,14,15] Genetic engineering also allows for greater flexibility in the design of a bispecific antibody in terms of size, valence, specificity, half-life, and biodistribution.[16] These bispecific antibodies represent many formats or technologies, including TrioMab,[17-19] bispecific T-cell engager (BiTE),[15,20] tandem antibodies (TandAbs),[21] immune-cell-mobilizing monoclonal TCRs against cancer (ImmTACs),[22,23] dual-action Fab (DAF),[24] IgG single-chain Fv fragments (scFv),[25] CrossMab,[26] "dock and lock" (DNL) antibodies,[27] dual variable domain IgG (DVD-Ig),[28-30] and nanobodies.[31]

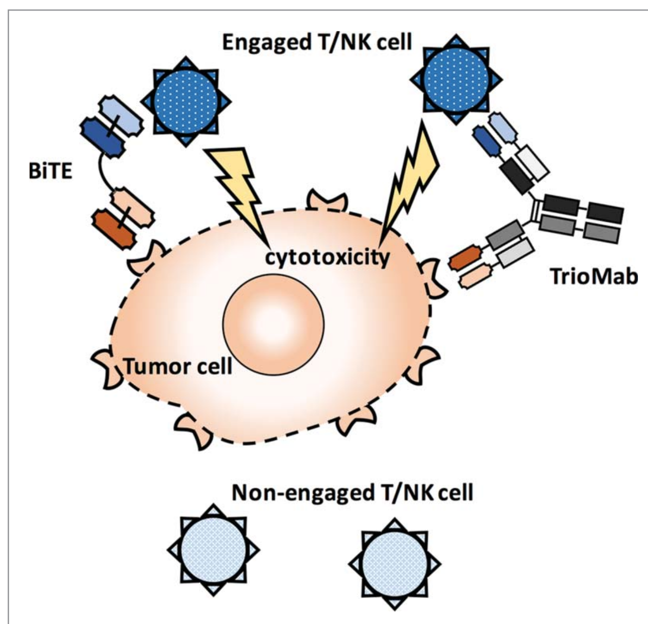

Figure 1. Mechanisms of action of bispecific antibodies. BiTE and TrioMab are shown here to demonstrate the tumor cell killing induced by bispecific antibodies. T cells or NK cells are recruited to the proximity of tumor cells by bispecific antibodies. Engaged T or NK cells will then attack tumor cells and lead to cytotoxicity, while non-engaged T cells or NK cells remain inactive toward the tumor cells.

On the basis of format, bispecific antibodies can be subdivided into 2 groups: IgG-like or bispecific fragment molecules (Fig. 2). IgG-like bispecific antibodies retain the structure of an IgG molecule with a functional Fc region. The Fc region facilitates purification and improves solubility and stability. Furthermore, the Fc region can induce antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity and can increase the serum half-life due to the large molecular size and FcRn-mediated recycling mechanism.[32] These features can be useful for certain therapeutic applications. In contrast, bispecific fragment molecules lacking Fc regions rely solely on their antigen-binding capacity for execution of their therapeutic activities. The smaller size of bispecific fragment molecules, however, can potentially enable better tumor tissue penetration and ensure stronger therapeutic effects.

The IgG-like bispecific format

This is a commonly used format of bispecific antibody. A bispecific antibody of the hybrid IgG format is monovalent for each antigen. Nevertheless, bispecificity can be achieved via fusion of either the amino or carboxy termini of either light or heavy chains with additional antigen-binding units (Fig. 2). The first approved bispecific antibody is catumaxomab (in 2009), which was developed for the treatment of malignant ascites in patients with EpCAM-positive tumors.[7,33] It is currently also being tested in phase I clinical trials against gastric cancer and phase I/II trials against epithelial cancer. Catumaxomab is produced by the fusion of a mouse hybridoma and rat hybridoma; this procedure results in a hybrid antibody of a rat IgG2b and murine IgG2a with

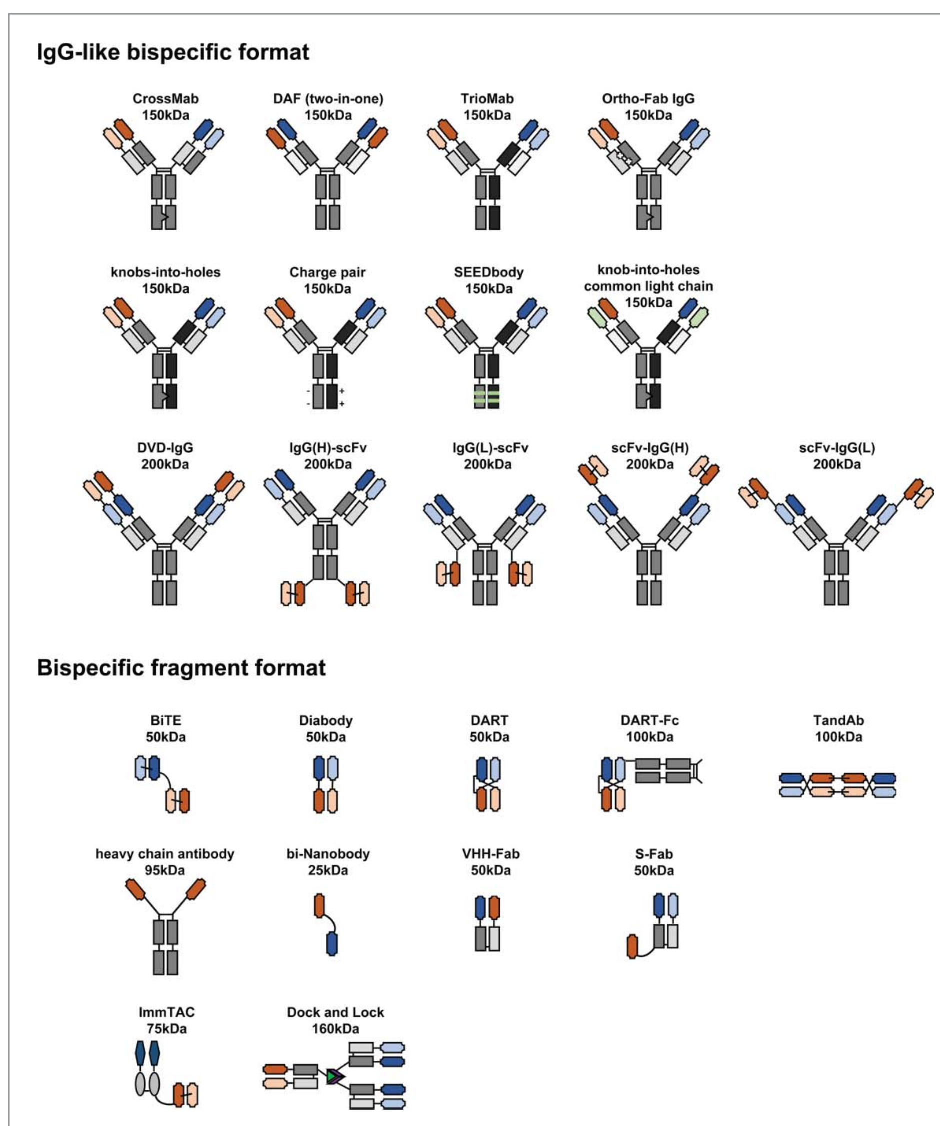

Figure 2. Diverse formats of bispecific antibodies. Heavy chains are shown in dark shades of black, gray, orange or green, while corresponding light chains are in lighter shades of the same colors. Peptide linkers are shown in thin black lines and engineered disulfide bonds by thin green lines. Heavy chain mutations are shown with dark gray triangles. Approximate molecular weights are estimated assuming ~12.5 kDa per immunoglobulin domains.

anti-CD3 and anti-EpCAM binding capacity, respectively.[17] The anti-EpCAM Fab fragment of catumaxomab can bind to tumor cells that express EpCAM, and anti-CD3 Fab of catumaxomab can recruit T lymphocytes to tumor cells. The Fc region of catumaxomab can bind to and activate Fcγ receptor-positive accessory cells, such as monocytes, macrophages, DCs, and NK cells.[34] Catumaxomab shows potent cytotoxicity toward ovarian carcinoma cells, preventing or reducing the accumulation of ascites.[7,18,33] Because catumaxomab is a mouse-rat hybrid IgG molecule, human anti-mouse or anti-rat antibody responses are observed in most patients.[6]

Several other bispecific antibodies have been created using a similar quadroma technology. For example, FTBA05[35] (which was designed to redirect T lymphocytes to B-cell lymphoma cells by targeting CD20) and ertumaxomab[36] (which was designed against tumor cells expressing HER2) are now in different phases of clinical development for patients with relapsed or refractory B-cell lymphoma or metastatic breast cancer, respectively.[37]

The manufacture of hybrid bispecific antibodies is a serious challenge because typical antibody populations that are secreted are heterogeneous. Additionally, light chains can mispair with a noncognate heavy chain. As a consequence, the desired bispecific antibody can be coexpressed with up to 9 unwanted, mispaired species.[38,39] In addition to the problems with the pairing of heavy chains and light chains, human anti-mouse antibody (HAMA) responses are another potential problem with this bispecific format.[16]

Homodimerization of the 2 heavy chains of IgG is mediated by the interaction between CH3 domains. To overcome the problem of unwanted heavy-chain paring, the "knobs-into-holes" strategy was developed.[40,41] The knobs-into-holes strategy utilizes a "knob" mutation (T366W) and pairing "hole" mutations (T336S, L368A, and Y407V) in the CH3 domains. The mutated CH3 domains favor heterodimerization of heavy chains over homodimer formation.[40,41] On the basis of the knobs-into-holes method, a variety of other strategies to increase heavy chain heterodimerization were proposed, such

as rational design of electrostatic steering mutations,[42,43] the use of alternative mutations,[44] and hybrid CH3 domains derived from IgG and IgA.[45] Although most bispecific antibodies are based on human IgG1 to generate IgG-like bispecific antibodies, some are based on other immunoglobulin isotypes including IgG2 and IgG4.[43,46]

In addition to heavy-chain pairing, another issue with bispecific antibodies in the IgG format is the problem with light-chain pairing. One way to circumvent this problem is to use a common light chain for both antibodies, which can be obtained by screening phage display libraries.[47] More recently, antibodies with a common light chain have been derived from transgenic mice with a single light chain.[48] Another way to overcome the problem with light-chain pairing is to express and purify the knob- or hole-containing half-antibodies separately in 2 types of host cells, followed by *in vitro* assembly into full bispecific antibodies.[43,49,50] A major advantage of the *in vitro* assembly strategy is its wide applicability to pre-existing antibodies, thereby reducing research and development costs. Additionally, the 2 different light chains usually enhance antigen-binding affinity and specificity of the resulting antibody.[48,51]

Another solution to the issues with heavy-chain and light-chain pairing is to fuse a second antigen-binding unit to the N or C terminus of either the heavy or light chains of the first parental monoclonal antibody to achieve both multivalence and bispecificity. In this case, antigen-binding units can be either single-chain Fc fragments (scFv) or single-domain antibodies (VL or VH).[51,52] A higher specific binding capacity can be attained due to the simultaneous binding to antigens with all variable domains.[30] DVD-IgGs are generated using this strategy. To create a DVD-IgG, the variable heavy chain domain (VH) and variable light chain domain (VL) from one parental monoclonal antibody are fused to the VH and VL, respectively, of another parental monoclonal antibody.[28,30] DVD-IgGs are bispecific and bivalent toward each antigen, with the potential of an extended range of valence and specificity.

Another efficient technical solution that simultaneously resolves the problem with light- and heavy-chain pairing in one host cell is the CrossMab method,[26] where correct pairings of the light chains are achieved via domain crossover with a heterodimerized heavy chain using the knobs-into-holes strategy.[47] The CH1 domain of one heavy chain is exchanged with the constant domain of the corresponding light chain (CL). For the domain crossovers, either the variable domains or the constant domains are swapped between light and heavy chains to create 2 asymmetric Fab arms.[53]

More recently, a combination of computational design and X-ray crystallography was used to introduce mutations into both the CH1-CL and VH-VL interface of the Fab fragments, resulting in orthogonal Fab interfaces, thus enforcing correct heavy chain–light chain pairing.[54] This design was used in combination with a heavy-chain heterodimerization strategy to facilitate efficient IgG production in a single host cell.[54]

Another alternative solution to the chain pairing problems is to use a single heavy or light chain and to engineer the variable domains to recognize 2 unrelated antigens.[51] The two-in-one or DAF platform takes advantage of the differential yet overlapping complementarity-determining regions (CDRs) as main contacts for each antigen.[24,55,56] The tetraspecific antibody FL518 combines 2 different DAF antibodies in CrossMab format and binds to HER2, VEGF, EGFR, and HER3 simultaneously,[57] showing *in vivo* antitumor activity superior to that of the 2 parental DAF antibodies.[57]

The bispecific-fragment format

Bispecific antibodies can be constructed without some or all of the constant domains of an antibody. The smaller size of such antibodies offers a possible advantage for better tumor tissue penetration over IgG-like format antibodies. Yet, the smaller size also shortens the serum half-life. A rapidly growing repertoire of bispecific fragment formats has been proposed and studied (Fig. 2). One common approach is to fuse 2 different scFv molecules in either the VH-VL or VL-VH orientation, with a short linker (1–10 amino acid residues) between the 2 scFvs[51] (although sometimes a certain orientation inhibits antigen binding[58]). Recombinant DNA technology allows for construction of a bispecific antibody in a variety of formats with great flexibility. A number of formats have been proposed, including BiTE,[15,20] TandAbs,[21] Diabody,[59] dual-affinity-retargeting format (DART),[60] and ImmTacs.[22,23]

A BiTE antibody is a 55-kDa recombinant protein composed of 2 scFvs, linked via a glycine-serine 5-amino-acid non-immunogenic linker.[61,62] BiTE was one of the first proposed formats of bispecific antibodies and is currently the most advanced in terms of clinical development. There are 2 scFvs; one is designed to bind to CD3 on T cells and the other to a tumor-associated antigen on tumor cells in a tandem scFv format,[63] which can redirect T cells to kill tumor cells directly. The BiTE strategy can be expanded via fusion of a third scFv fragment, resulting in a trivalent and/or trispecific antibody.[64,65] BiTEs are produced as recombinant proteins in CHO cells.[61] Although BiTEs have a short half-life in serum (because they lack the Fc region and have a relatively small molecular size), they are effective and can exert specific tumor cell cytotoxicity at picomolar concentrations *in vitro*.[66] BiTEs can induce antigen-dependent polyclonal T-cell activation, potent T-cell mediated cytotoxicity, and T-cell proliferation,[61,62,67] which are likely related to the efficient activation of the T-cell receptor (TCR) complex.[62]

The BiTE antibody blinatumomab (AMG 103, MT103) is approved by the US Food and Drug Administration for treatment of patients with Philadelphia chromosome-negative precursor B-cell acute lymphoblastic leukemia (B-ALL).[8,9] Blinatumomab is composed of 2 scFvs, one targeting CD19 and one against CD3, with equilibrium dissociation constants (K_D) of 10^{-9} and 10^{-7} M, respectively.[66] After engagement of T cells via the anti-CD3 domain, blinatumomab creates a structural immune synapse with $CD19^+$ cells,[68] leading to T-cell activation and cytotoxicity toward $CD19^+$ cells.[69,70] Both $CD4^+$ and $CD8^+$ T cells can be induced to proliferate and engage in cytotoxicity without previous stimulation.[71] Perforin and granzyme expression is increased after engagement with the complex, which leads to cell lysis of the $CD19^+$ cells.[71] Blinatumomab has been shown to be effective, as patients with non-Hodgkin's lymphoma who receive blinatumomab as a single agent show apoptosis of $CD19^+$ cells[70] at a much lower dose compared with the anti-CD20 mAb rituximab.[72] However, blinatumomab requires continuous intravenous infusion for several

weeks by means of a portable pump to ensure continuous activation of T cells against target cells due to the rapid clearance of blinatumomab. Other BiTEs that are in various stages of development, targeting tumor-associated antigens such as EGFR, EpCAM, fibroblast-activating protein α, prostate stem cell antigen (PSCA), HER2, carcinoembryonic antigen (CEA), ephrin A2 (EphA2), MET, and CD33.[6,62]

A similar approach, called diabody, involves coexpression of 2 different scFv fragments by means of a short peptide linker that allows for the heterodimerization of the 2 complementary fragments.[59] Mutations can be introduced into the VL–VH interface to favor heterodimerization over homodimerization to improve the yield of the desired format.[73,74] The introduction of an engineered disulfide bond into a diabody can improve stability. The same approach to improve stability is used in the DART format[60] as well as a dimeric tetravalent tandem diabody called "tandAb," which is produced from 2 pairs of VH and VL domains connected via a polypeptide linker.[75,76]

DARTs are diabody-like molecules where the VH region of the first antibody is linked to the VL of the second antibody, and the VH of the second antibody is linked to the VL of the first antibody.[6] As noted above, DARTs are further stabilized via introduction of disulfide bonds.[77] A CD19-CD3 DART (MG011), in comparison with a bispecific antibody of identical specificity and similar structure (blinatumomab), was found to be significantly more potent in redirected $CD19^{+}$ cell killing assays *in vitro*.[77] As with blinatumomab, no activation of T cells by the DART was observed in the absence of engagement with targeted $CD19^{+}$ cells. The greater affinity and higher association rate for CD19 and CD3 of a DART, as well as the fixed orientation of the 2 binding domains, may contribute to the superior clinical performance of MGD011.[78] The fixed orientation of the 2 binding domains may also increase the number of productive engagements between the effector and target cells.[78]

MGD006, a DART that binds to cell surface protein CD123 and CD3 simultaneously,[79] showed potent activity in preclinical evaluation toward myeloid leukemia[79] and is now in a phase I clinical trial for patients with relapsed or refractory acute myeloid leukemia.[6] Another DART, MGD007, was designed to retarget T cells to $GpA33^{+}$ gastrointestinal tumor cells. Unlike MGD006, MGD007 is fused to an Fc fragment, a conventional protein engineering strategy that prolongs serum half-life.[80,81,6]

Another bispecific strategy is a DNL. A DNL construct involves the natural interaction between the dimerization and docking domain (DDD) of cAMP-dependent protein kinase A and the anchoring domain (AD) of A-kinase anchor protein (AKAP).[47,54] Two types of modules, the first one, a homodimer containing the DDD (fused with an Fab directed against the first antigen) and the second one containing the AD (fused to another Fab to the second antigen) are generated and then combined to form noncovalent complexes. Disulfide bonds are then formed between cysteine residues incorporated into the DDD and AD sequences to form a trivalent molecule.[27,78] Therefore, a DNL construct is composed of one Fab-AD and 2 Fab-DDD moieties.[82]

ImmTACs

ImmTACs, i.e., immune-cell-mobilizing monoclonal TCRs against cancer, are soluble bispecific agents that comprise an anti-CD3 scFv linked to an affinity-matured TCR that recognizes target MHC-peptide complexes.[22,23] The ImmTAC technology offers an opportunity to retarget T cells to intracellular tumor proteins presented as MHC-peptide complexes.[51]

Like other bispecific retargeting strategies, ImmTACs also utilize an anti-CD3 scFv arm of nanomolar affinity to target effector T cells. Unlike the other approaches, ImmTACs recognize tumor cells through an engineered high-affinity TCR (usually a monoclonal TCR) rather than through an antibody fragment. ImmTACs trigger T-cell activation through a natural steric and biological immune synapse.[22,23] The monoclonal TCR on ImmTACs can target intracellular tumor-associated antigens via recognition of MHC-peptide complexes (on the cell surface) that are rarely accessible to antibodies.[83] This strategy expands the spectrum of tumor-specific targets, with the potential to reduce off-target toxicity.[84] When in contact with tumor cells, ImmTACs specifically bind to a defined MHC-peptide complex displayed on the cell surface via high-affinity TCR-based recognition. Then, the CD3 arm recruits polyclonal T cells, thus, leading to the formation of an immune synapse and eventual killing of the tumor cells.[22]

Many ImmTACs have been developed, including those targeting gp100 (a melanocyte differentiation antigen), MAGE-A3 (a cancer neoantigen expressed in a wide array of tumors), Melan-A/MART-1 (a lineage-specific antigen expressed by metastatic melanomas), and NY-ESO-1 (a cancer neoantigen expressed in multiple myeloma).[83] Currently, the most advanced ImmTACs program, IMCgp100, is in phase II trials in patients with malignant melanoma.

Retargeting of NK cells

Aside from T cells, NK cells also play a crucial role in the recognition and eradication of tumors. NK cells have also been explored as targets for bispecific antibodies. To engage NK cells, the CD16 marker on NK cells is frequently used. AFM13, a tetravalent bispecific TandAb (CD30/CD16A), was developed recently.[85] AFM13 has 2 binding sites for the NK cell marker CD16A and 2 for CD30, which is a cell surface marker of Hodgkin's lymphoma cells. AFM13 was shown to induce stronger cytotoxicity toward tumor cells than an optimized anti-CD30 IgG or a bivalent bispecific CD16A/CD30 diabody.[85] Because of its larger molecular size (100 kDa), AFM13 exhibited a significant increase in serum half-life over BiTEs. AFM13 is now in phase II clinical trials for patients with Hodgkin's lymphoma, and in a phase I clinical trial for $CD30^{+}$ lymphoma. An alternative NK cell-retargeting bispecific antibody was constructed in the format of bispecific killer engagers (BiKEs) and showed potent cytotoxicity.[86,87] In a BiKE, the anti-CD3 arm in a BiTE construct is replaced with an anti-CD16 humanized scFv arm to recruit NK cells. Recently, to take advantage of the stimulation and proliferation capacity of IL-15,[80] a trispecific killer engager (TriKE) construct was proposed using IL-15 to link the 2 antibody arms for the purpose of boosting anti-tumor activity.[88]

Heavy-chain-only antibodies

To reduce the heavy chain–light chain problem, the VH or VL alone can be engineered to bind an antigen.[90-92] Such a VH or

VL can be used as a building block for bispecific antibodies. However, a better strategy is to use single-domain antibodies. A single-domain antibody is derived from natural camel heavy-chain-only antibodies (HCAbs).[93] The variable domain of the HCAbs is referred to as the VHH, also known as a single-domain antibody or nanobody. The VHH is the structural and functional equivalent of the Fab fragment of conventional antibodies[94] and the smallest fully functional, naturally derived antigen-binding fragment. Nanobodies have been shown to have several properties that makes them promising building blocks for antibody engineering, including small molecular size (15 kDa), high expression level, and high stability and solubility *in vitro*.[28,95,96] The CDR3 of camel VHHs forms an extended loop covering the lipophilic site; this property results in improved solubility.[97,98] Moreover, nanobodies were demonstrated to be as specific as regular antibodies.[99]

The major concern with nanobodies is their immunogenicity in humans. However, as nanobodies are small, undergo rapid renal clearance, and their amino acid sequences are highly similar to that of human VH, no significant immunogenicity has been observed in mice or humans injected with nanobody-containing constructs.[100-102] A humanization strategy has also been developed to further decrease the immunogenicity, which involves humanization of 12 out of the 14 amino acid residues that differ from the corresponding sequence in human IgG.[103]

The small size of nanobodies makes them amendable to bulk production in bacterial cells.[99] In general, nanobodies are cloned behind a secretion signal for bacterial expression. The proteins are produced in the periplasm where formation of disulfide bonds is possible. A yield of several milligrams per liter of culture can be achieved in the lab with a simple culture flask.

The relatively low molecular weight generally results in a better tissue bio-distribution, as well as access to epitopes on some targets that are difficult to access with antibodies of the IgG format.[104] The low molecular weight, however, may also cause rapid renal clearance, which could hamper therapeutic success.

Bispecific antibodies can be constructed by linking 2 nanobodies (specific to 2 antigens) in tandem via a peptide linker.[105] Apart from tandem VHHs, one alternative to achieve bispecificity is to fuse one VHH single-domain antibody with the CH1 and another with the CL, in a single Fab fragment.[106] One research group combined an anti-CD16 VHH in this Fab format to recruit NK cells with an anti-HER2 VHH to target HER2$^+$ tumor cells.[107] This Fab-like bispecific antibody showed increased potency against HER2$^+$ tumor cells in comparison with trastuzumab.[107] S-Fab, a hybrid of human Fab with a VHH, was constructed by linking a single-domain anti-CEA VHH to a human anti-CD3 Fab.[108] S-Fab can be efficiently expressed and purified from bacteria and showed excellent stability in serum and potent antitumor activity *in vitro* and *in vivo*.[108] Another NK cell-retargeting bispecific antibody called BiSS was generated in the binanobody format recently.[89] BiSS can be efficiently expressed and purified from bacteria. It utilizes an anti-CD16 VHH arm to recruit NK cells and an anti-CEA VHH arm to target CEA-expressing tumor cells. These studies suggest that the flexibility of VHHs will enable their broad use in bispecific antibodies.

Concluding remarks

As the next generation of antitumor strategies, bispecific antibodies have received much attention because of their unique mechanism of action and potent tumoricidal effect. The success of blinatumomab as monotherapy for B-ALL added a great deal of excitement to this field. Numerous bispecific retargeting antibody candidates are now being tested in clinical trials,[6,51] with a plethora of others in preclinical studies.

Future advances in bispecific antibody technology will be focused on the development of new antibody formats to accommodate the complexity of tumor biology. Beyond bispecific antibodies, more extensive research into multispecific and multivalent antibodies is also anticipated. Because the majority of current bispecific antibodies target known tumor cell surface antigens, discovery of new targets is also urgently needed, including novel tumor cell surface antigens and intracellular tumor antigen-associated peptide-MHC complexes, to increase efficacy and reduce adverse effects of bispecific antibodies. Of equal interest is the rise of a new class of bispecific antibody targeting tumor-associated antigens and checkpoints to tumor-site specifically reverse immune suppression and release effector cells in-check. It should be noted that tumor biology is complex and diverse. Genome sequencing has revealed the heterogeneous nature of tumors, even within the same patient, and the wide variety of mutations that may be present in cancer cells.[109] It is likely that most bispecific retargeting antibodies alone may not be as efficacious as blinatumomab is against B-ALL, which is considered less complex than solid tumors. A combination of different therapeutics to mount a multifaceted attack on a tumor is expected to result in long-lasting cancer-free survival.

Abbreviations

AD	anchoring domain
B-ALL	B-cell acute lymphoblastic leukemia
BiKE	bispecific killer engager
BiTE	bispecific T-cell engager
CML	chronic myeloid leukemia
CDR	complementarity-determining region
DC	dendritic cell
DDD	dimerization and docking domain
DNL	“dock and lock”
DAF	dual-action Fab
DART	dual-affinity-retargeting format
DVD-IgG	dual variable domain IgG
ImmTACs	immune-cell-mobilizing monoclonal TCRs against cancer
CL	light chain
NK	natural killer
scFv	single-chain Fv fragment
TCR	T-cell receptor
TandAb	tandem antibody
VH	variable heavy chain domain
VL	variable light chain domain

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The authors declare that they have no conflicts of interest.

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