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REVIEW ARTICLE

Expanding the Boundaries of Biotherapeutics with Bispecific Antibodies

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Abstract

Bispecific antibodies have moved from being an academic curiosity with therapeutic promise to reality, with two molecules being currently commercialized (Hemlibra® and Blincyto®) and many more in clinical trials. The success of bispecific antibodies is mainly due to the continuously growing number of mechanisms of actions (MOA) they enable that are not accessible to monoclonal antibodies. One of the earliest MOA of bispecific antibodies and currently the one with the largest number of clinical trials is the redirecting of the cytotoxic activity of T-cells for oncology applications, now extending its use in infective diseases. The use of bispecific antibodies for crossing the blood–brain barrier is another important application because of its potential to advance the therapeutic options for neurological diseases. Another noteworthy application due to its growing trend is enabling a more tissue-specific delivery or activity of antibodies. The different molecular solutions to the initial hurdles that limited the development of bispecific antibodies have led to the current diverse set of bispecific or multispecific antibody formats that can be grouped into three main categories: IgG-like formats, antibody fragment-based formats, or appended IgG formats. The expanded applications of bispecific antibodies come at the price of additional challenges for clinical development. The rising complexity in their structure may increase the risk of immunogenicity and the multiple antigen specificity complicates the selection of relevant species for safety assessment.

Key Points

The success of bispecific antibodies is mainly due to the new biologies they enable.

Some promising applications (i.e., improved tissue delivery) still offer ample potential for further growth.

The additional binding specificities present a more difficult scenario for safety assessment experiments.

Immunogenicity in patients may provide a critical barrier for developing highly engineered antibody formats.

1 Introduction

Monoclonal antibodies (mAbs) have revolutionized therapeutics for a broad range of disease indications [1]. In oncology, they can work through a variety of mechanisms of action [2]; for example, directly blocking the interaction between an angiogenic factor and its receptor [3], or blocking the mitogenic interaction of two membrane tyrosine kinases [4]. In addition, mAbs can also induce antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-mediated cytotoxicity (CMC) [5], or stimulate the patient's immune system to produce an anti-tumor response [6]. Similar to the advancement in the therapeutics field propelled by mAbs, the advent of bispecific antibodies is greatly expanding the therapeutic potential of antibodies in general. Bispecific antibodies are able to target two different epitopes simultaneously; these can either be on the same antigen or on two different antigens. In some applications, bispecific antibodies do not provide a functional advantage over the combination of the corresponding monospecific antibodies; however, they offer a simpler and more cost-effective development path than two individual drugs, which would require two separate

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manufacturing processes [7] as well as filing on the safety of each antibody component separately [8]. However, the biggest impact of bispecific antibodies in the pharmaceutical field is due to the fact that they enable novel mechanisms of action not accessible to monospecific antibodies. This type of bispecific antibodies has been referred to as 'obligatory bispecifics' [9] as the mechanisms of action depend on the physical connection of the antibodies with the two distinct specificities. The novel biologies enabled by bispecific and multispecific antibodies are the main thrust for their success, and thus the central focus of this review. In the following sections, we briefly review the different antibody formats in the context of design considerations for the different applications. More in-depth reviews on the structural aspects of the different antibody formats can be found in previous excellent reviews [9–11]. In addition to an up-to-date review of the growing applications of bispecific antibodies, we also discuss actual challenges and potential future hurdles for the clinical advancement of bispecific antibodies.

2 Origin and Evolution of Bispecific Antibody Formats

The current success of bispecific antibodies is only possible because the initial limitation for their efficient production has been overcome. The main problem for producing bispecific antibodies using wild-type IgG sequences was that the random association of chains led to the combinatorial associations of the two heavy and light chains, creating up to ten different products. Early attempts to produce bispecific antibodies relied on the conjugation of antibody fragments or on the fusion of two different hybridomas to generate a quadroma. These approaches were suitable for research purposes but not for clinical applications as the source material was not easily scalable [12]. The earliest engineering solution to the random association of the heavy chains without using linkers or chemical conjugation was the so-called 'knobs-into-holes' strategy, where two sets of sterically complementary mutations in the C_H3 domain alter the association interface such that heterodimerization is greatly favored over the formation of homodimers [13]. Another early strategy to produce bispecific antibodies and overcoming the initial limitations was the fusion of antibody fragments. The first described fragment-base format was the so-called 'diabody' [14], where two different chains with crossed-over variable domains (V_HA-V_LB/V_LA-V_HB) associate to form the bispecific molecule. As the benefits of bispecific antibodies have become clearer, these two initial strategies have evolved into an ever-growing plethora of related formats [9, 10]. The remarkable explosion in diversity of all bispecific antibody formats has been fueled not only by the pursuit of more efficacious and/or more developable formats, but also by the need to achieve freedom to operate without infringing on intellectual property. In parallel with the increase in diversity in bispecific antibody formats, the number of patents filed for bispecific antibodies has been growing in recent years [15]. In spite of the variety in antibody formats, some fundamental overarching criteria should be considered when developing a bispecific antibody. They are discussed below.

3 Considerations for Selecting a Bispecific Antibody Format

3.1 Developability

Generation of therapeutic antibodies with a reasonable cost requires that the engineered proteins express at high levels. This is perhaps the earliest critical attribute that novel platforms need to fulfill in order to be viable. Reduction of the cost of goods is a consideration that is also driving the adoption of IgG-like formats compatible with expression in a single cell (some of these are discussed later in the text) or the exploration of processes that allow expression in a single fermentor [16]. Another important aspect is the purification strategy, as a platform that requires a very complex purification scheme would be disadvantageous over other alternatives with a simpler process. Associated with purification, the proper characterization of the desired product and potential contaminant species is important to guarantee the robustness of the production process and the quality of the final product. Depending on the format, the potential formation of different unwanted species could be more difficult to detect and quantify, presenting a bigger challenge for analytical groups. For example, single-cell expression of a bispecific IgG with two different heavy chains and two different light chains, although it simplifies downstream process and reduces costs, may lead to the formation of contaminants with very similar biochemical properties to the intended product and may require advanced methods for their detection [17]. The chemical and physical stability of the protein is also important, as poor stability may compromise the activity as well as increase the risk of immunogenicity. Physical stability has been a limitation, for example, for the development of some single-chain variable fragment (scFv)-containing bispecific antibody formats due to their intrinsic propensity to aggregate [18]. Several approaches have been developed to overcome this limitation, such as introducing a stabilizing disulfide bond, grafting the complementarity determining regions (CDRs) onto a stable framework, CDR engineering [19], or by swapping kappa and lambda framework regions [20]. In one published example, the use of scFvs with frameworks preselected for increased stability translated into better biophysical properties for the corresponding mAb-scFv bispecific antibody [21]. Introducing

novel functions in an IgG usually requires significant modifications of the IgG structure that often reduces the stability of the resulting protein. For example, one strategy to create bispecific antibodies is the recruitment of an antigen binding site in the C_H3 domain of huIgG1 by diversifying the structural loops. Using this approach, antibodies binding $\alpha_5\beta_3$ [22] and HER2 [22] through the C_H3, called FcAbs, have been described. The alteration of the structural framework, however, resulted in proteins with reduced thermal stability that had to be recovered through a directed evolution protocol [23] in order to improve its developability.

3.2 Pharmacokinetics

Most applications of bispecific antibodies require a long half-life in circulation to support sustained drug exposure compatible with infrequent dosing. In some instances, however, a short half-life may provide a safety advantage because in the event of a drug-induced adverse event, the therapeutic can be quickly eliminated from circulation. For example, blinatumomab is a bispecific T cell engager (BiTE) associated with neurological adverse events that are reversible upon discontinuation of dosing. The fast clearance of the format is a benefit in this context [24]. Pre-targeting strategies for imaging and radiotherapy also benefit from a short half-life of the targeting antibody. In these applications a bispecific antibody capable of binding the radionuclide and a tumor-assocated antigen is given to patients first. Once the antibody has cleared from circulation, leading to a high tumor-to-blood and tissues ratio, a peptide loaded with the radionuclide is administered. Antibody formats with short half-lives lead to high tumor-to-blood concentration ratios faster than antibody formats with longer half-lives [25, 26] and thus have an advantage in this application. In addition to the common factors affecting the PK properties of mAbs such as charge, glycosylation, and polyreactivity [27], other aspects related to the format in general may have more profound effects on the PK profile of bispecific antibodies. IgGs usually have a long half-life in serum due to the binding of the Fc portion to the FcRn receptor that prevents endosomal degradation and recycles the endocytosed molecules back to serum [28]. Some of the bispecific formats lack the Fc fragment and thus inherently clear more rapidly from circulation.

3.3 Effector Function

The Fc region of an IgG mediates different cytotoxic mechanisms, such as activation of the classical pathway of the complement through interacting with C1q [29], as well as activation of cytotoxicity by natural killer (NK) cells and phagocytosis by macrophages through interaction with different Fcγ receptors on the cell surface. Retaining effector functions could be required for some applications; for example, antibodies against infectious agents mediating an increase in pathogen uptake [30]. On the contrary, interactions of the Fc with immune cells or the complement may lead to undesired toxic effects in other cases, as discussed later in the section on bispecific antibodies for crossing the blood–brain barrier (BBB). A remarkable advance in the ability to eliminate or modulate these interactions allows for a tailored design of the effector function that best serves the intended application [31]. For example, mutations have been identified that promote the hexamerization of IgGs, leading to a more efficient recruitment of C1q [32, 33]. Also, mutations that increase binding to C1q [34–36] or FcγRIIIa [37–39] leading to enhanced complement-dependent cytotoxicity and ADCC, respectively, have been described.

3.4 Immunogenicity

Administration of therapeutic antibodies in humans may trigger the production of anti-drug antibodies (ADAs), which may have unwanted consequences. ADAs may reduce or abrogate the activity of the antibodies by blocking their function or by removing them from circulation, or they can lead to toxic responses. The factors involved in determining immunogenicity of a given therapeutic are diverse and complex [40], but it is a well established immunological observation that the lower the homology a protein has to the endogenous counterpart, the higher the immunogenic potential, as the chances of containing a T-cell epitope increase. Therefore, when developing or selecting a bispecific antibody format, it is preferable to minimize the differences with a natural IgG. The potential risks of increased immunogenicity associated with highly engineered bispecific formats and some mitigation efforts through the development of reliable methods for the early assessment of immunogenicity are discussed in a separate section below.

Each bispecific antibody format has advantages and disadvantages in terms of the fundamental considerations described above. They also vary in the extent to which they have been adopted in the pharmaceutical field, ranging from single academic groups to a wide number of companies, as well as in how advanced they are in clinical trials. In the following sections we focused the review on those formats with wider use and those that are being tested in the clinic. For a comprehensive snapshot of bispecific antibody format variants, the reader is referred to previous reviews [9, 10]. Also, bispecific formats can be developed based on a variety of alternative scaffolds [41, 42], but these are outside the scope of this review.

4 IgG-Like Formats

This is a group of related engineered solutions rendering a final product that resembles a natural IgG. Because they contain the Fc fragment, IgG-like formats have long serum half-life, usually a desirable attribute for most biological applications. Moreover, if an application so requires, the half-life can be modulated by using a series of mutations in the Fc that alter the binding affinity to FcRn, extending or shortening the half-life of an IgG [43]. In contrast to other formats that contain scFvs, IgG-like formats with minimal deviation from the native sequence show good physical stability properties, which simplifies the development processes and reduces the risk of immunogenicity caused by aggregates [44]. Importantly, IgG-like formats lack long stretches of non-native sequences and have none or few exposed non-native residues, reducing the immunogenic potential. While most IgG-like formats rely on engineered surfaces to drive the preferential formation of the bispecific molecules, a few rely on the purification process to isolate the bispecific molecules from side products; some examples of each class are reviewed below.

4.1 IgG-Like Formats Engineered for Preferential Bispecific Formation

Some of the most widely adopted formats are shown in Fig. 1. After the initial publication describing the 'knobs-into-holes' engineering by Ridgway et al. [13], other research groups have exploited mutations in the Fc creating steric complementation [45–48] or charge repulsion/attraction [49, 50] as a driver for heterodimerization. The use of heterodimeric C_H3 solves the correct pairing of the heavy chains, while four types of strategies have been devised to solve the heavy chain–light chain pairing problem: (a) in vitro annealing, (b) common light chain, (c) crossing over

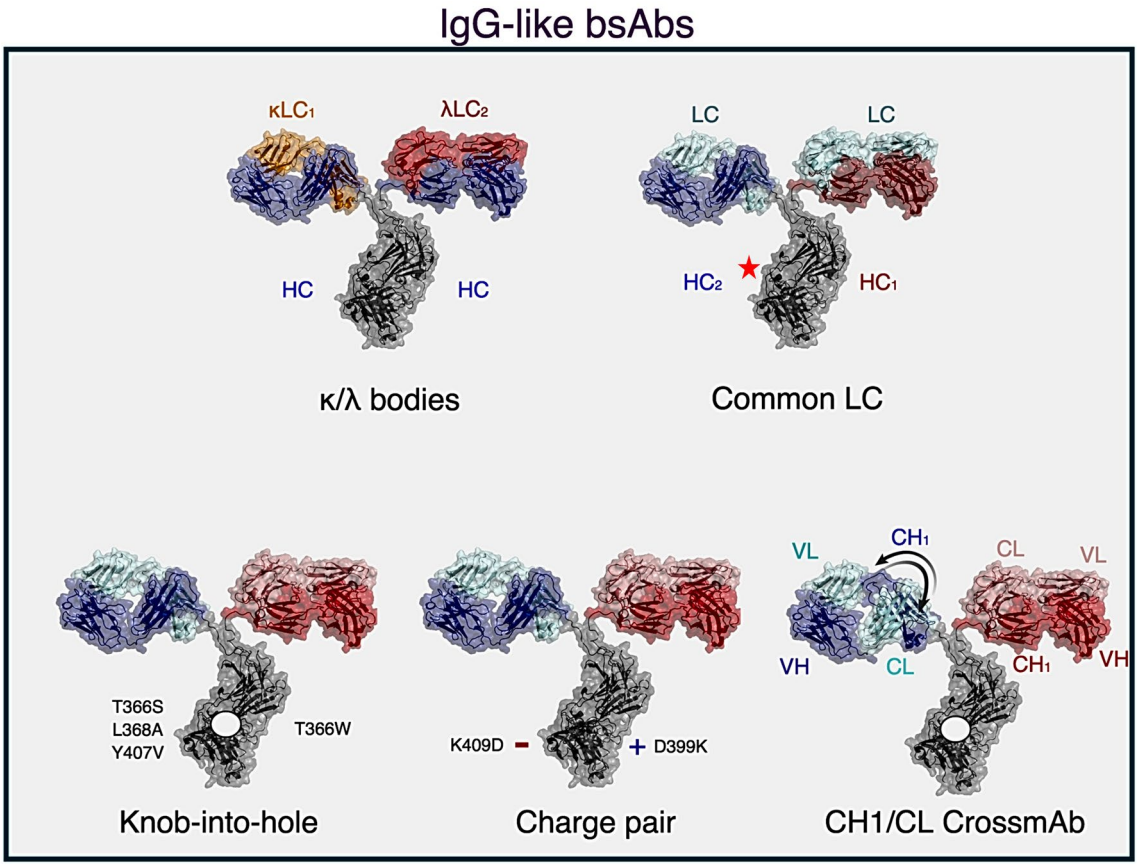

Fig. 1 Selected IgG-like bispecific antibody formats. 'κλ bodies' (Novimmune) contain a common heavy chain (HC) and employ the difference in light chain (LC) backbones for purifying the bispecific antibody from contaminant products. The 'common LC' format scheme represents the format used by Regeneron; the *red star* symbolizes the star substitution in one of the heavy chains. In the 'knob-into-hole' format (Genentech), the three mutations creating the 'hole' and the single mutation creating the 'knob' are indicated. In the 'charge pair' antibody format (Amgen), the mutations within the C_H3 domain that favor heterodimeric HC association are indicated. The 'CrossMAb' (Roche) format employs the knob-into-hole approach for correct HC pairing, as well as a domain swap to enable orthogonal LC–HC pairing. The scheme depicts a CrossMAb where the C_L and C_H1 domains have been swapped

part of the heavy and light chain domains, and (d) steric/charge mutations driving orthogonal heavy chain–light chain association. In the in vitro annealing strategy, two sets of half antibodies or bivalent antibodies with heterodimeric C_H3 domains are expressed and purified independently and then assembled in vitro into the bispecific molecule. The annealing procedure is performed under mild conditions that preserve the heavy chain–light chain associations so no mispairing occurs. This process is currently used in bispecific antibody formats developed by Genentech [51, 52] and Genmab [46, 53], among others. The common light-chain approach requires the use of two different antibodies that share the same light chain [54] (Fig. 1); this can be achieved by screening a phage library [55, 56] or by immunizing transgenic mice with a fixed single light chain [57]. This is the process being used by Chugai, Regeneron, and Merus for their respective antibody platforms. The crossing over strategy has been used by Roche to create the CrossMAb platform. The technology involves creating hybrid molecules between the heavy and light chains for one of the two parental antibodies in the bispecific molecule [58]. Considering the heavy chain of the antibody to be crossed over, there are three possible ways of generating the hybrid molecules: crossing over the two domains making the antigen-binding fragments (Fab) (replacing both V_H and C_H1 for V_L and C_L1), or just the variable domain or the first constant domain. Figure 1 presents an example where the C_H1 and C_L domains have been swapped (indicated by arrows). Finally, different mutations that drive the desired association between the heavy and light chains either by steric complementation, electrostatic steering or by introducing engineered disulfide bonds have recently been described [59–62]. Except for the in vitro annealing method that requires two separate cell cultures, the other three approaches when used in combination with heterodimeric C_H3 domains allow for the expression of all chains in a single cell, which reduces costs and simplifies manufacturing.

4.2 IgG-Like Formats Engineered for Facilitated Purification

A different approach used by Regeneron utilizes heterodimeric C_H3 domains not to drive the preferential association of heterodimers but to facilitate their purification from a pool of heavy-chain homo- and heterodimers. One of the heavy chains carries two mutations (referred to as 'star' substitution)(Fig. 1) that abrogates binding to Protein A, so during Protein A purification the star/star homodimer species (Fc*/Fc*) flow through the column and the heterodimers are purified from wt/wt homodimers (Fc/Fc) using a pH gradient or step elution [63]. Because resins based on recombinant Staphylococcal Protein A are able to bind the Fab of some antibodies (i.e., V_H3 framework), the process initially worked only on a subset of bispecific antibodies and required additional purification steps for antibodies containing V_H3 domains [63]. The recent development of a resin utilizing a protein A lacking the domains responsible for binding to VH3, and with an optimized base matrix, allowed the adoption of a single protein A column steps for the removal of homodimers from all class of antibodies [64]. The co-expression of heterodimeric Fc chains requires the use of a common light chain, which Regeneron has solved in the case of the anti-CD3/CD20 bispecific antibody by taking the light chains of a collection of fully human anti-CD3 clones and paring them with the heavy chains of a panel of anti-CD20 antibodies and selecting those anti-CD3 light chains that supported CD20 binding [65]. Unlike mutations in the C_H3–C_H3 interface that are not solvent exposed, the star substitution is accessible on the surface; however, in silico analysis predicted the lack of T-cell epitopes that could increase the immunogenicity of the molecule. Similar to the approach used by Regeneron that relies on purification, Novimmune has developed a bispecific antibody format called 'κλ bodies' that introduces no mutations in the Fc or Fab regions. This format requires using a common heavy chain and two different light chains, one κ, one λ [66]. The co-expression of the three different chains produces a mixture of bispecific and monospecific antibodies from which the bispecific is isolated through three tandem affinity purifications: protein A, Kappa-Select and Lambda-Select. Although the selection of a common heavy chain may require significant upfront engineering effort and the purification process could be costly, the final product contains no non-native polypeptide sequences, which minimizes the immunogenic potential. It is worth mentioning that the two approaches, engineering for preferential bispecific formation and for facilitated purification can be combined. Chugai, for example, employs electrostatic steering to drive heterodimerization of the heavy chains, and in the case of ACE910, the bispecific antibody mimetic of Factor VIII, mutations were also introduced to facilitate purification by ion exchange chromatography (IEX). Two initial bispecific antibody leads showed no significant differences in the isoelectric points with respect to the homodimeric species, but after the introduction of charged substitutions in the variable region of the heavy chains, differences in the isoelectric points greater than 0.5 pH unit were achieved that supported efficient separation by IEX [67]. This type of strategy could be useful in multispecific antibody formats where an increased complexity in the molecule may lead to undesired mispaired species with very similar biochemical properties to the corrected assembled molecule.

5 Fragment-Based Bispecific Antibodies

5.1 V_H/V_L-Based Formats

Antibody fragments have been used to produce bispecific antibodies (some are shown in Fig. 2) for a few decades. Initially Fabs were crosslinked chemically [68], or fused to dimerization tags [69]. Only later the use of antibody fragments to enable the production of bispecific antibodies without a conjugation or assembly step was described. The first format described was diabodies, which consist of two crossed-over scFv ($V_HA - V_LB/V_HB - V_LA$) that can only form dimers due to the use of a short linker separating the two variable domains in each chain [14]. Homodimers are usually formed when expressing diabodies, and strategies using engineered disulfide bonds and complementary surfaces have been described to improve the percentage of heterodimeric diabodies [70]. Another commonly used format is the tandem scFv, where all four variable domains are connected in a single polypeptide chain. A common problem with scFv is the weak binding affinity for the V_H–V_L pair [71] which, in the absence of stabilizing constant domains, makes the molecule prone to dissociation and the formation of aggregates. Some solutions to this problem that have been explored include the selection of more stable scFv frameworks [21] or the engineering of cysteines for the formation of an intra-Fv disulfide bond that stabilizes the V_H–V_L interaction [72]. Seeking an alternative but related solution to the stability problem, Johnson et al. designed a format called DART (Dual Affinity Re-Targeting proteins), which basically is a diabody where the chains contain engineered cysteine residues that allow formation of an inter-Fv disulfide bond [73] (Fig. 2). In addition to stabilizing the molecule, the disulfide bond creates a more rigid, compact structure than a regular diabody and tandem scFv, as well as having a different geometry: while the binding sites in classic diabodies are located in opposing ends facing away [74, 75] (in a tail-to-tail configuration), in DARTs the binding sites are closer, facing ~90° away from each other [76]. More recently, Egan et al. described a strategy to increase the stability of scFvs that involves replacing a region of the V_L kappa domain with the corresponding sequence from a germline V_L lambda domain, reporting improved thermal and storage stability for the chimeric scFv domains [77]. TandAbs is a bispecific tetravalent format described in 1999 [78] and further developed by researchers from Affimed. Four variable domains in the configuration $V_HA/V_LB/V_HB/V_LA$ are tethered by short linkers that prevent the formation of intra-chain Fv (Fig. 2). Non-covalent dimerization in a head-to-tail organization reconstitutes all four Fvs [79].

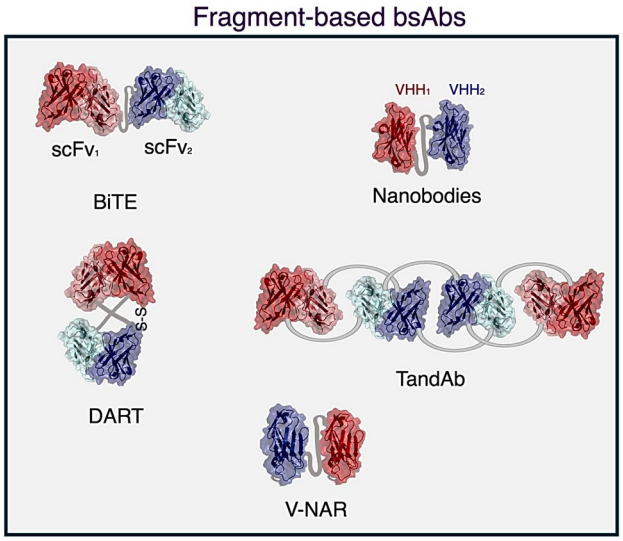

Fig. 2 Selected fragment-based bispecific antibody formats. The 'BiTE' (bispecific T-cell engager) format (Amgen) consists of two scFvs connected with a Gly/Ser peptide linker. DARTS (Dual Affinity Re-Targeting proteins, MacroGenics) are diabodies containing an inter-Fv disulfide for increased stability that results in a structure that is rigid and compact. 'TandAbs' (Affimed) are dimers of scFvs containing the $V_HA/V_LB/V_HB/V_LA$ domain organization where short linkers favor the correct assembly of the Fvs. The resulting molecule is bivalent for each specificity. Single domain antibodies like V_HH and shark single variable new antigen receptor domain antibody fragments (VNARs) can be easily fused to create bispecific 'nanobodies' and 'VNARs'

5.2 Single-Domain Bispecific Antibodies

This is the most recent addition to the fragment-based class of format. Single-domain antibodies are derived from immunoglobulins that contain a single variable domain, which are found naturally in some camelids (camels, llamas, alpacas and vicuñas) [80] and cartilaginous fishes (i.e., sharks) [81]. The single-domain antibodies from camelids are known as V_HH or nanobodies, whereas the ones from sharks are known as V-NAR (variable new antigen receptor domain) (Fig. 2). The rising interest in this type of antibodies resides in their multiple benefits. Single-domain antibodies are easy to produce in different hosts, they are highly soluble, stable and with comparable affinities to regular antibodies. The small size makes them well suited for imaging applications, where fast penetration into the tissues and fast clearance from circulation is desirable [82, 83]. One key feature of camelid nanobodies is that they have a CDR3 loop of more variable length (2–28 residues) than the human CDR3. Nanobodies with a long, protruding CDR loop can access cavities or clefts sometimes not accessible to regular antibodies [83]. Targets with cryptic epitopes where nanobodies have been used successfully include ion channels [84], the active site of enzymes [85] and G protein-coupled receptors [86], with all three groups containing proteins with important

therapeutic potential. To minimize immunogenicity in humans, different strategies to humanize nanobodies [87, 88] and V-NARs [89, 90] have been described. An alternative to humanizing single-domain antibodies with animal origin is to 'camelize' human variable domains to turn them into single-domain antibodies. By replacing amino acid residues in the framework of the V_H domain [91–93], and sometimes also requiring substitutions in the CDRs [94, 95], it is possible to obtain monomeric human V_H domains. A number of strategies have been developed over time to further increase the stability and solubility of human V_H or V_L domains [96, 97].

6 Appended IgGs or Fcs

This is a group of formats where an antibody fragment is fused to the regular structure of an IgG or Fc fragment. The fragments may be fused to either the N- or C-terminus end of the heavy (appended IgG and Fc) or light chains (appended IgGs), and the fused fragments could be Fvs, scFvs, Fabs, single domain antibodies or alternative scaffolds; thus, the number of potential different formats is quite large. Some of the formats in this group are shown in Fig. 3. Unlike IgG-like formats that are typically monovalent for each specificity, most appended IgGs are more commonly bivalent for at least one of the specificities. The multivalency in some cases is irrelevant for the mechanism of action, while in other cases the increased valency may contribute to the activity of the molecule. The latter is discussed in more detail in the Sect. 7.5 below. Conversely, symmetric appended IgGs would not be a suitable format for those applications where multivalency is not desirable. For example, MET is a tyrosine kinase involved in cell proliferation and protection against apoptosis that is deregulated in some types of cancer [98]. Upon binding to its ligand, the hepatocyte growth hormone, MET dimerizes and becomes activated. Although non-agonistic bivalent antibodies against MET have been described [99], most antibodies against MET are agonistic and their use in bivalent bispecific formats may produce an agonistic rather than antagonistic molecule. Other applications of bispecific antibodies where it is important to preserve monovalent binding are T-cell engagement and crossing the BBB using the transferrin receptor, which are discussed later in the text.

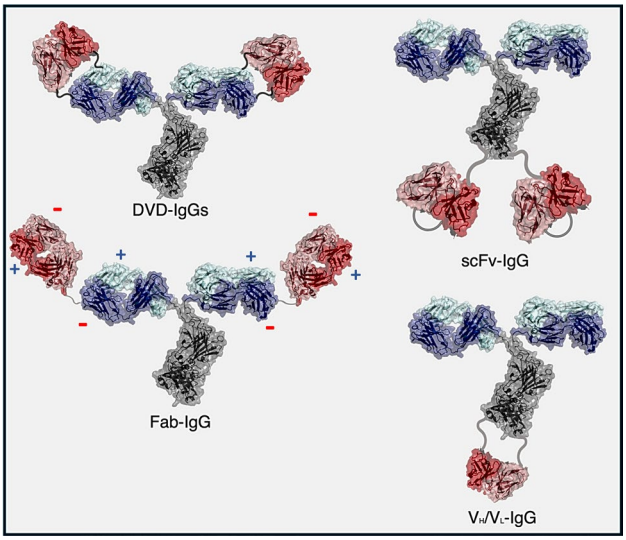

Fig. 3 Selected appended-IgG bispecific antibody formats. Shown are the symmetric fusion of Fv fragments to generate 'DVD-IgGs' (AbbVie) and the asymmetric fusion of an Fv domain to create an 'V_H/V_L-IgG' [238], bivalent for the mAb specificity and monovalent for the specificity conferred by the Fv. The symmetric fusion of scFvs to the C-termini of the HCs generate a 'scFv-IgG' (MedImmune), a bivalent molecule for each binding specificity. The represented 'Fab-IgG' contains symmetric fusion of Fab fragments in the N-termini of the HCs. Indicated are charge mutation pairs required to direct the cognate association of the heavy and light chains

Similar to IgG-like formats, appended IgGs also contain the Fc fragment and their serum half-lives are comparable to regular IgGs. One difference with IgG-like formats is the presence of linkers and non-natural junctions that could potentially increase the immunogenicity of this type of molecule. scFv fragments are prone to aggregation, so formats that rely on this fragment may require more upfront engineering efforts to stabilize the fragment or downstream formulation optimization. One interesting feature of appended IgGs is that they possess multiple chain termini where fragments can be fused, which allows the generation of the same bispecific with different geometries. Many examples (one of them is mentioned in the scFv Sect. 6.1) are described in the literature, illustrating the effect of varying the architecture of an appended IgG on the activity of the resulting molecules. Thus, finding the best configuration for a given application may require screening different antibody format designs. As mentioned above, the diversity of formats within this group is remarkable; in the following sections we review some of the formats, grouping them according to the nature of the appended fragment. Tables 1, 2, 3, 4 and 5 detail the bispecific antibodies currently in clinical development, grouped by mechanism of action (see also Sect. 7).

6.1 Fv-IgG

DVD-IgGs or dual-variable fragment immunoglobulins were described by researchers at AbbVie in 2007 [100]. The architecture of DVD-IgGs is similar to a typical IgG with added antigen-binding variable regions (V_H/V_L), each connected to the corresponding heavy or light chains via peptide linkers (Fig. 3). Three types of linkers with variable length have been utilized: the 'elbow' regions linking the V_L to C_L or V_H to C_H1, the regions linking C_H1 to C_H2

Table 1 Selected bispecific antibodies recruiting effector cells in clinical development

MOA	Drug	Sponsor	Targets	Trial	Format	Indication	Phase	References
T-cell engager	REGN-1979	Regeneron	CD20/CD3	NCT02290951	IgG	NHL, CLL	I	[65]
T-cell engager	ERY-974	Chugai	Glypican3/CD3	NCT02748837	IgG	Solid tumors	I	[50]
T-cell engager	RG-7828/ BTCT4465A (mosunetuzumab)	Genentech	CD20/CD3	NCT02500407	IgG	NHL, CLL	I	[224]
T-cell engager	NA	Genmab	CD20/CD3	NA	IgG (DuoBody)	B-cell malignancies		
T-cell engager	JNJ-63709178	Janssen	CD123/CD3	NCT02715011	IgG (DuoBody)	AML	I	[225]
T-cell engager	JNJ-64007957	Janssen	BCMA/CD3	NCT03145181	IgG (DuoBody)	MM	I	[226]
T-cell engager	JNJ-64407564	Janssen	GPRC5D/CD3	NCT03399799	IgG (DuoBody)	MM	I	
T-cell engager	AFM11	Affimed	CD19/CD3	NCT02106091 NCT02848911	TandAb	NHL ALL	I I	
T-cell engager	AMV564	Amphivena	CD33/CD3	NCT03144245	TandAb	AML	I	
T-cell engager	Blinatumomab	Amgen	CD19/CD3		BiTE		Marketed	
T-cell engager	AMG-330	Amgen	CD33/CD3	NCT02520427	BiTE	AML	I	
T-cell engager	AMG-420	Amgen	BCMA/CD3	NCT03287908	BiTE	MM	I	
T-cell engager	AMG-596	Amgen	EGFRViii/CD3	NCT03296696	BiTE	Glioblastoma	I	
T-cell engager	Flotezumab	MacroGenics	CD123/CD3	NCT02152956	DART	AML/MDS	I	
T-cell engager	MGD-014	MacroGenics	HIV/CD3	NCT03570918	DART	HIV	I	
T-cell engager	AMG-673	Amgen	CD33/CD3	NCT03224819	BiTE-Fc	AML	I	
T-cell engager	AMG-701	Amgen	BCMA/CD3		BiTE-Fc	MM	I	
T-cell engager	AMG-757	Amgen	DLL3/CD3	NCT03319940	BiTE-Fc	SCLC	I	
T-cell engager	MGD-009	MacroGenics	B7-H3/CD3	NCT02628535	DART-Fc	Several	I	
T-cell engager	MGD-007	MacroGenics	gpA33/CD3	NCT02248805	DART-Fc	CRC	I	
T-cell engager	RG-6026	Roche	CD20/CD3	NCT03075696	1-Fab-IgG (2 + 1)	NHL	I	[215, 227]
T-cell engager	CD123/CD3	Roche	CEA/CD3	NCT02324257 NCT02650713	1-Fab-IgG (2 + 1)	CEA + solid tumors	I	[107, 228]
T-cell engager	XmAb14045	Xencor	CD123/CD3	NCT02730312	Fab/scFv-Fc	AML	I	
T-cell engager	XmAb13676	Xencor	CD20/CD3	NCT02924402	Fab/scFv-Fc	FcNHL/CLL/ SLL	I	
T-cell engager	XmAb18087	Xencor	STTR2/CD3	NCT03411915	Fab/scFv-Fc	NET/GIST	I	
T-cell engager	GBR 1302	Glenmark	HER2/CD3	NCT02829372	Fab/scFv-Fc (BEAT)	HER2 + cancer	I	[229]
T-cell engager	GBR 1342	Glenmark	CD38/CD3	NCT03309111	Fab/scFv-Fc (BEAT)	MM	I	
NK-engager	AFM13	Affimed	CD30/CD16	NCT01221571 NCT02605650 NCT03192202	TandAb	HL + Keytruda CL	I I Ib/II2	[146]

AI autoimmune diseases, *ALL* acute lymphocytic lymphoma, *AML* acute myeloid leukemia, *BiTE* bispecific T-cell engager, *CEA* carcinoembryonic antigen, *CL* cutaneous lymphoma, *CLL* chronic lymphocytic leukemia, *CRC* colorectal carcinoma, *DART* dual affinity re-targeting proteins, *Fab* antigen-binding fragment, *GIC* gastrointestinal cancer, *GIST* gastrointestinal stromal tumor, *HL* Hodgkin lymphoma, *MDS* myelodysplastic syndrome, *MM* multiple myeloma, *MN* metastatic neoplasms, *NA* not available, *NET* neuroendocrine tumor, *NHL* non-Hodgkin lymphoma, *NK* natural killer, *NSCLC* non-small cell lung cancer, *scFv* single-chain variable fragments, *SCLC* small cell lung cancer, *SLL* small lymphocytic lymphoma

of huIgG1, and glycine/serine linkers [101]. The positioning of the variable domains as the outer or inner domains can be critical for optimal binding of both antigens, which can be dependent on the size and cellular localization of the antigens [102]. In addition, the length of the linkers can also be optimized to fine tune the affinities of both the outer and inner variable domains [103]. For immunology applications, the symmetric tetravalent form of the platform has been used, while asymmetric, monovalent DVD formats have been used in T-cell redirecting [104].

Table 2 Selected bispecific antibodies blocking two ligands or two different pathways in clinical development

Area	Drug	Sponsor	Targets	Trial	Format	Indication	Phase	References
Ophthalmology	RG-7716 (RO6867461)	Roche	VEGF/Ang-2	NCT03038880 NCT02699450	CrossMAb (1 + 1)	AMD DME	II II	[230]
	BI 836880	Boehringer Ingelheim	VEGF/Ang2	NCT02674152	Nanobody	Solid tumors		
Oncology	ABT-165	AbbVie	DLL4/VEGF	NCT01946074 NCT03368859	DVD	Solid tumors CRC	I II	
Oncology	JNJ-61186372	Janssen	EGFR/MET	NCT02609776	IgG (DuoBody)	NSCLC	I	[150, 231]
Immunology	NA	Eli Lilly	BAFF/IL17	NA	NA	AI	I	
Immunology	ABT-981	AbbVie	IL-α/IL-β	NCT02384538 NCT02087904	DVD	Hand OA Knee OA	IIa IIa	[232] [233, 234]
Immunology	ALX-0761(M1095)	Avillion	IL-17A/IL-17F	NCT03384745	Nanobody	Psoriasis	II	
Oncology	MGD013	MacroGenics	PD-1/LAG-3	NCT03219268	DART-Fc	Metastatic neoplasm	I	

AI autoimmune diseases, *AMD* age-related macular degeneration, *CRC* colorectal carcinoma, *DART* dual affinity re-targeting proteins, *DME* diabetic macular edema, *DVD* dual variable domain, *EGFR* epidermal growth factor receptor, *NA* not available, *NSCLC* non-small cell lung cancer, *OA* osteoarthritis, *VEGF* vascular endothelium growth factor

Table 3 Selected bispecific antibodies promoting the association of membrane-associated proteins in clinical development

MOA	Drug	Sponsor	Targets	Trial	Format	Indication	Phase	References
Hormone mimetic	RG-7992 (BFKB8488A)	Genentech	FGFR/Klothoβ	NCT02593331 NCT03060538	IgG	Type II diabetes	I I	[158]
Hormone mimetic	RG-6013 (ACE910) (emicizumab)	Chugai	Factor iXa/X		IgG	Hemophilia A	Marketed	[235]
Clustering	ZW-25	Zymeworks	Her2 (biparatopic)	NCT02892123	IgG (Azymmetric)	HER2 + cancers	I	

Table 4 Selected bispecific antibodies for crossing the BBB in clinical development

Area	Drug	Sponsor	Targets	Trial	Format	Indication	Phase	References
Lysosomal storage disorders	AGT-181	ArmaGen	InR/IDUA	NCT03053089	mAb-Enzyme fusion	MPSI	II	[172]
	AGT-182	ArmaGen	InR/IDS	NCT02262338	mAb-Enzyme fusion	MPSII	I	

BBB blood–brain barrier, *IDUA* α-L-iduronidase, *IDS* iduronate 2-sulfatase, *InR* insulin receptor, *MPSI* mucopolysaccharidosis I

Table 5 Selected bispecific molecules improving tissue-specific delivery or function in clinical development

Area	Drug	Sponsor	Targets	Trial	Format	Indication	Phase	References
Oncology	ABBV-428	AbbVie	CD40/mesothelin	NCT02955251	Undisclosed	Solid tumors	I	
Oncology	PRS-343	Pieris	CD137/HER2	NCT03330561	Anticalin-IgG4	HER2 + tumors	I	[236]
Oncology	M7824	Serono	PDL1/TGF-β	NCT02517398	aPDL1 mAb-TGF-βRII fusion	Solid tumors	I	[199, 237]

6.2 Fab-IgG

The fundamental CrossMAb architecture can be used to add fragments and create more complex molecules, reviewed in detail by Klein et al. [105]. The format more extensively used to date is a heterodimeric/asymmetric trivalent antibody (also referred to as '2 + 1') [106] with the Fab appended to the N-terminal end of the heavy chain, which is being used as a platform for T-cell engaging applications [107]. Examples of symmetric tetravalent molecules with the Fab fused to the C-termini of the HC are also available (referred to as '2 + 2') [108]. Other formats fusing Fabs to the N-termini of the heavy chains and directing the correct association of the light chains by mutations in the CH1-CL interface have been described [106]. In addition, expressing a Fab as a single-chain fused to a heterodimeric IgG is another strategy that has been used to create an asymmetric molecule without introducing mutations to solve the heavy chains–light chains association [109].

6.3 ScFv-IgG

ScFvs can be fused to different ends of an IgG in a symmetric (such as the example shown in Fig. 3) or asymmetric fashion. Although most studies have been done with only one of the multiple possible symmetric configurations, a few studies have compared bispecific antibodies with different attachment sites of the scFv fragments. Bispecific antibodies combining two epitopes on the HIV coreceptor molecules CCR5 showed little effect of the scFv fusion site on the activity of the resulting antibodies [110]. In contrast, DiGiandomenico et al. found that a specific scFv fusion configuration led to a more efficacious drug against *Pseudomonas* [111]. Thus, the existence of a positional effect is not universal and is likely to depend on the structure and function of the target. However, it could be important when making a new scFv-IgG fusion to test multiple configurations as not all of them might be equally active. The use of an asymmetric format where one arm is a regular Fab-Fc and the other arm is scFv-Fc fusion is a strategy that allows expression of all three chains in a single cell, circumventing the light chain–heavy chain mispairing problem, provided a heterodimeric Fc is used. Glenmark [112] and Xencor [113] are two companies implementing this approach. Interestingly, Glenmark developed the heterodimeric Fc based on structural data from the association between the α and β chains of the T-cell receptor.

6.4 Single-Domain Ab-IgG

Examples of this type of fusion exist in the context of building upon a well characterized mAb. For example, Osiannix is using a VNAR against the transferrin receptor to develop their platform for crossing the BBB. In one application, they are fusing the VNAR to rituximab to deliver it into the brain for the treatment of multiple sclerosis and brain cancer [114]. Unlike other examples using appended IgGs, in this particular case multiple VNAR-IgG fusion configurations were tested, and four of them were found to have acceptable brain penetration activities [114].

6.5 BiTE-Fc, DART-Fc, Single-Domain Ab-Fc

Because of the short half-life that limits the application of many fragment-based bispecific antibodies, different solutions to improve their pharmacokinetic properties are being explored; one of these is to fuse them to the Fc using symmetric or asymmetric designs. Depending on the application, a symmetric or asymmetric design is preferred. For example, MacroGenics is developing two bispecific antibodies to block checkpoint inhibitors, MGD013 and MGD019 (Table 2), where multivalency for each antigen could be advantageous. In these two bispecific antibodies, one of the two chains making the DART is fused to homodimeric Fc, thus resulting in a tetravalent bispecific molecule. MacroGenics is also advancing a series of T-cell engager DARTs fused to Fc (Table 1). Because monovalent binding of CD3 is desirable, for this application the design is asymmetric: the three-chains construct utilizes Fc domains carrying the 'knob' or 'hole' mutations where one of the heterodimeric Fcs is fused to one of the chains of the DART, resulting in a bispecific molecule monovalent for each specificity. DART-Fc molecules have shown improved half-lives ranging between 70 h [115] and 105 h [76] in mice, an improvement over the regular version of DARTs with half-life values ranging from 2.4 to 3.6 h [73]. An extended half-life of ~ 6.5 days has also been observed in cynos [116]. BiTEs (bispecific T-cell engagers) are the fragments with the shortest half-life among bispecific fragments, thus they are the ones that benefit the most from half-life extending efforts. Recently, an Fc-fusion version of BiTEs has been published, reporting an improved half-life of ~ 210 h [117] or 112 h [118]. In this case as well, an asymmetric design is required to preserve the monovalency of the anti-CD3 domain. Most typically, bi- or multi-specific antibodies based on single-domain antibodies include an albumin-binding domain as the strategy to extend their half-life in circulation [119, 120]. However, at least one example exists where fusion to the Fc was used to extend the half-life of a nanobody. Li et al. created an NK-recruiting antibody by fusing two V_HHs (against CD16 and CEA) to a heterodimeric Fc, in a format named SS-Fc [121]. The PK profile of the bispecific molecule was not reported, but it caused tumor regression in mouse xenograft models [121].

7 Mechanisms of Action Enabled by Bispecific Antibodies

The success of bispecific antibodies is mainly due to the fact that they enable mechanisms of action not accessible to monospecific antibodies. They find applications in diverse therapeutic areas such as oncology, neurology, infectious diseases, metabolism, and regenerative medicine. The applications for bispecific antibodies have multiplied rapidly and most likely there are still untapped areas to be discovered. Some distinctive mechanisms of action enabled by bispecific antibodies are shown in Fig. 4 and described below.

7.1 Redirecting the Cytotoxic Activity of Effector Cells

This was the earliest application of bispecific antibodies and it continues to be one of the widest pursued uses today (Table 1) [68]. Here one arm of the bispecific antibody is designed to bind a specific antigen expressed on tumor cells while the other arm targets a receptor capable of activating the effector cell upon crosslinking. Effector cells (macrophages, neutrophils, NK cells, granulocytes or cytotoxic T cells) express different types of activating receptors, and a specific population can be recruited by carefully selecting the targeted trigger receptor [122].

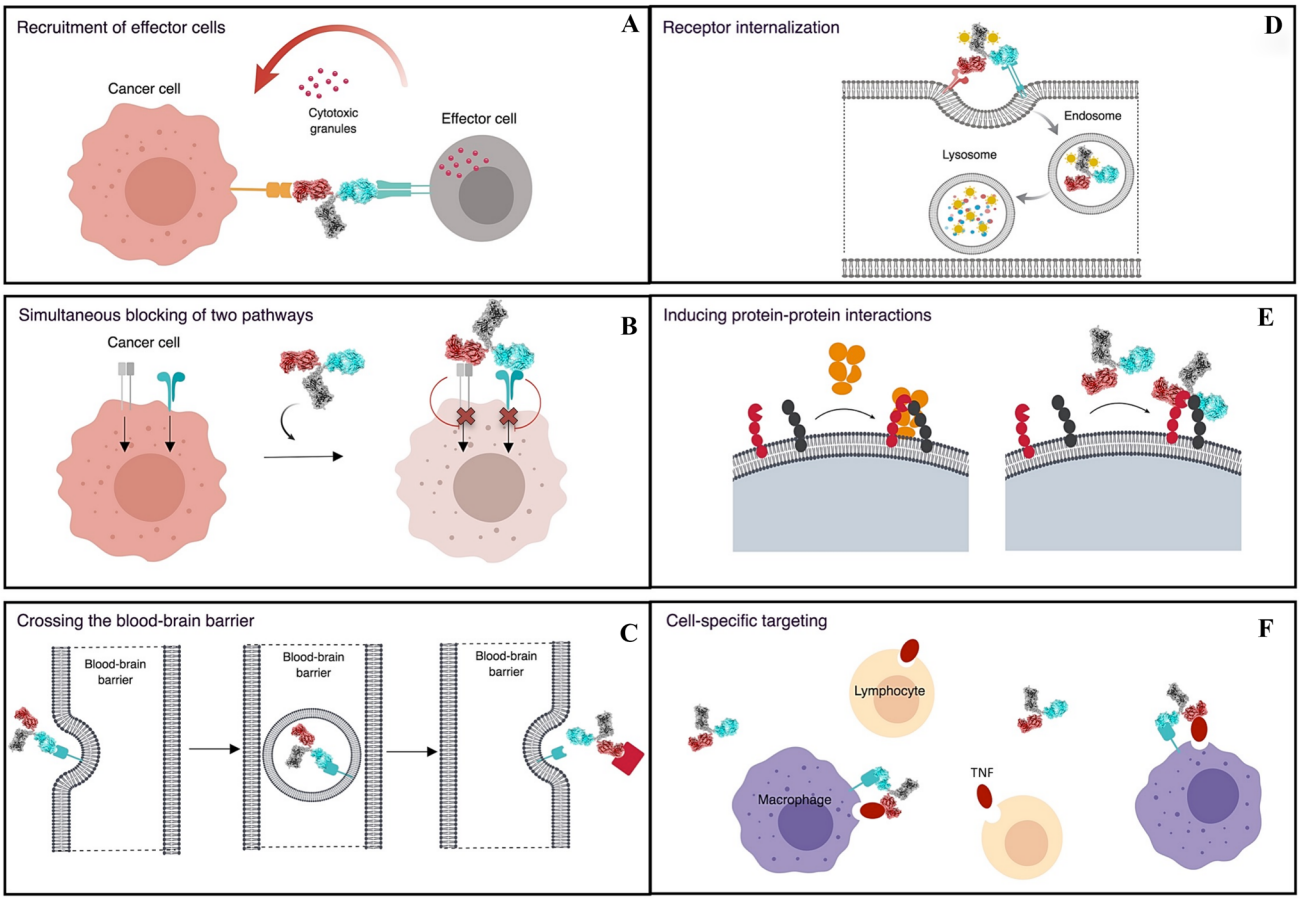

Fig. 4 Mechanisms of action enabled by bispecific antibodies. (A) Redirecting effector cells for cytotoxicity. The bispecific antibody is designed to simultaneously engage a cancer and effector cell (i.e., T cells) resulting in effector cell activation and cancer cell death. (B) Simultaneous blockade of two pathways. Targeting of two receptors on cancer cells (i.e., EGFR and MET) can result in a more potent inhibition of cell growth. (C) Transcytosis across the blood–brain barrier (BBB). One arm of the bispecific antibody recognizes a receptor that promotes shuttling across the BBB (e.g., transferrin receptor) and the other targets a pathway in the brain involved in neuropathology. (D) Hyperclustering of receptors for internalization. Bispecific antibodies can induce hyperclustering of receptors and antibody internalization, which can be exploited to increase delivery of antibody–drug conjugates. (E) Forced interaction of membrane or membrane-associated proteins. Bispecific antibodies can be used to mimic factors involved in forming productive membrane protein complexes (i.e., factor VIII to enable clotting). (F) Tissue-specific delivery. Targeted delivery of bispecific antibodies to tissues can reduce liabilities from systemic administration (i.e., targeting TNF on macrophages)

Redirected cytotoxic activity has been shown with bispecific antibodies recruiting all effector cells including macrophages [123]; however, recruitment of T cells is far more widely used (Table 1) because of their proliferation ability and potent cytotoxicity. For this application, CD3ε, a component of the T-cell receptor (TCR), is the most commonly used target, although examples of antibodies targeting the α/β chains also exist [124]. Other surface proteins present on T cells have been explored as well but with lower killing efficiencies in general. For example, bispecific antibodies engaging CD28 [125] and CD2 [126] have been reported to have some cell killing efficacy. A wide variety of antibody formats and targets have been evaluated for this application, and as of today > 25 T-cell recruiting antibodies targeting both hematological malignancies as well as solid tumors are in various stages of clinical development [127] (Table 1). In general, monovalent engagement of CD3 is preferred to avoid clustering the TCR in the absence of binding to target cells, but some formats with bivalent binding to CD3 are being explored. Efforts to compare across different bispecific antibody formats for T-cell redirection have been limited in scope—diabody vs BiTE [128], DART vs BiTE [129], BiTE vs TandAb [130], and BiTE vs Fab-C_H2-scFv (Fabsc) [131] have been compared for in vitro potency in the context of a single target. Many factors such as antibody affinity [132], antigen density and size [133], epitope location [134], antibody size and distance between paratopes [135], multivalency for the target [136, 137], and flexibility of the antibody domains [129] affect the efficacy of T-cell killing. Thus, comparison of the same formats in the context of different antigens may produce a different rank order in some cases. More importantly, other considerations like pharmacokinetics, safety, and immunogenicity may dictate preferences between different formats. For instance, a very potent molecule may be desirable against a target with expression absolutely restricted to tumors or with expression in non-essential tissues, whereas a format not so potent but more selective towards cells expressing a high density of the target may be preferable for targets showing some expression in normal tissue. For example, CD20 is a target for B-cell malignancies with expression restricted to B cells. Because patients tolerate depletion of normal cells, development of potent T-cell engagers against CD20 could be beneficial if devoid of increased toxicities. An asymmetric Fab-IgG CrossMAb bivalent for CD20 and monovalent for CD3 (2 + 1) has been developed by Roche. The presence of two anti-tumor Fab enables avidity, which at least for an anti-CD20 T-cell bispecific antibody (TCB), conferred higher potency compared with the standard monovalent bispecific (1 + 1) [215]. In regard to the use of effector-less or effector-competent formats, it is worth noting that catumaxomab (anti-EpCAM/CD3), the first approved and now discontinued bispecific antibody, was a T-cell recruiting bispecific antibody with an effector competent Fc. The Fc was able to activate CD16 + NK cells and dendritic cells in vitro [138] and in vivo [139], likely linking the adaptive and innate immunity [140]. Most if not all T-cell engager bispecific antibodies being advanced in the clinic today, however, either lack the Fc completely or have an effector-attenuated version of it to avoid Fcγ receptor-mediated clustering and potentially activating T cells in an antigen-independent manner. Nevertheless, data from experiments with catumaxomab highlight the benefits of simultaneously stimulating the innate immune system, so combinations of T-cell engagers with other agents stimulating accessory immune cells could be an area of interest.

The extreme potency of T-cell redirecting bispecific antibodies is a double-edge sword: although it could be convenient when engaging tumor cells, it could be a problem when the tumor antigen is also expressed at lower levels in normal tissue. A new class of T-cell engagers that are inactive in circulation, and only become activated in the tumor, is being developed by different companies using various strategies. For example, CytomX has described an anti-EGFR/anti-CD3 antibody where the two paratopes are 'masked' by peptides that are connected to the N-termini of the heavy and light chains by linkers containing cleavage sites for tumor-enriched proteases [141]. In a poster presented at an American Association for Cancer Research conference, CytomX reported the masked format showed anti-tumor activity in mouse xenografts expressing the linker-cleaving proteases, while the maximum tolerated dose in monkeys was 60-fold higher than the unmasked version [141]. In the pursuit of expanding the therapeutic index of T-cell engagers, Amunix has developed the platform ProTIA, consisting of a BiTE fused to an XTEN molecule via a linker containing tumor-expressed proteases. The XTEN moiety is not only intended to extend the half-life of the molecule, but it is also proposed to increase the preferential permeation into the tumor tissue as well as preventing the formation of an effective synapse between T cells and target cells. A ProTIA targeting EpCAM and CD3 was shown to have a half-life of 32 h in mice and it was capable of producing 97% of tumor growth inhibition in Xenograft models [142].

The format that revitalized the interest for bispecific antibodies was the tandem single-chain Fv, also known as BiTE because of the impressive preclinical and clinical results. An anti-CD3/anti-CD19 BiTE (blinatumomab) entered phase I trials in 2001 and was approved by the FDA in 2014 for the treatment of some cases of acute lymphoblastic leukemia (commercialized as Blincyto®). Because of the short half-life of the molecule, the patients receive the drug by constant pump-infused IV administration. To date, blinatumomab has been tested in a variety of indications with variable but overall high response rates and cytokine release syndrome and reversible side effects related to the central nervous system

[143]. Although not as represented as T-cell engagers, bispecific antibodies recruiting NK cells are also being pursued as potential therapeutics. They have been shown to have good activities both in vitro and in vivo [144, 145]. One potential benefit over T-cell engagers is that activation of NK cells leads to lower levels of proinflammatory cytokines than activation of T cells, thus reducing the risk of a cytokine storm. AFM13 is the bispecific antibody for redirecting NK cells that is most advanced in clinical development (Table 1). Affimed has recently announced AFM13 is well tolerated and effective, both as a monotherapy or in combination with anti-PD1 [146].

In addition to the oncological application, the recruitment of effector cells has been explored more recently for the control of human pathogenic viruses like human immunodeficiency virus (HIV) [115, 147], and cytomegalovirus (CMV) [148]. This application has implications for the treatment of other severe infectious diseases beyond HIV and CMV.

7.2 Simultaneous Blockade of Multiple Pathways or Proteins

Many biological functions are complex, and the result of the integration of different signals. Therefore, the blockade of a signal pathway is often not enough to completely shut off a given biological process. Bispecific or multispecific antibodies can also be used to simultaneously block two different pathways or proteins implicated in a biological function (Table 2). As mentioned previously, the use of bispecific antibodies is preferable over a combination of mAbs because they offer a simpler and less costly development path. Although these are not 'obligatory bispecific' molecules, they account for a good number of bispecific antibodies being explored (some shown in Table 2). Cell growth, angiogenesis, and inflammation are multi-factorial functions of therapeutic interest where bispecific antibodies are used. Examples of the blockade of two receptor tyrosine kinases (RTKs) that are involved in cell growth include the targeting of HER2 and HER3 [149], epidermal growth factor receptor (EGFR) and MET [150] and insulin growth factor receptor (IGFR1) and EGFR [151]. Efforts in the neovascularization field are targeting the vascular endothelium growth factor (VEGF) in combination with angiopoietin 2 (ANG2) [152] or delta-like 4 (DLL4) [153] for more pronounced inhibitions. In a diversity of immunological diseases like rheumatoid arthritis, psoriasis, asthma, and Crohn's disease, bispecific antibodies have been used to inhibit simultaneously two inflammatory cytokines like IL4 and IL3 [154], interleukin 6 receptor and tumor necrosis factor α (TNFα) [155], IL17a and TNFα [156], or two different chemokine receptors [157]. The lasting anti-tumor effects observed with different antibodies against checkpoint inhibitors [6] has prompted the development of bispecific antibodies with dual immunomodulator specificity. Because all the targets are expressed on T cells, bispecific antibodies targeting two different checkpoint inhibitors may show an avidity effect, increasing the residency time and leading to a more pronounced effect. Several companies have different combinations using various antibody formats, some shown in Table 2.

Similar to the more robust tumor growth inhibition obtained by targeting multiple pathways, in infectious diseases the use of antibodies towards different epitopes may increase the breadth of neutralization. Recently Xu et al. described the generation of trispecific antibodies targeting different epitopes recognized by broadly neutralizing antibodies. The format utilized is called cross-over dual variable Ig-like (COVD-IgG) [208], a type of appended IgG format. The trispecific antibody showed higher potency and breadth than the parental antibodies and gave complete immunity to macaques exposed to a mixture of simian human immunodeficiency virus (SHIV) [209]. Because many infectious agents have the ability to mutate rapidly, the combination of agents attacking different protein surfaces essential for the function of the virus could reduce the chance of mounting simultaneous escape mechanisms. Similar approaches could be effective against dengue virus [210] and influenza [211].

7.3 Forced Interaction of Membrane or Membrane-Associated Proteins

This approach has diverse uses, such as the activation or inhibition of a signaling pathway or conferring a property of one protein to a second protein (Table 3). This strategy has been used to develop an anti-allergic antibody that brings the activating receptor FcεRI into contact with the inhibitory receptor FcγRIIb on the surface of mast cells, inhibiting their activation [55]. In some cases, the induced protein–protein interaction promotes a function rather than inhibiting it. For example, a bispecific antibody mimics the hormone fibroblast growth factor 21 (FGF21) by bringing the fibroblast growth factor receptor 1 (FGFR1) and the co-receptor Klotho-β in close proximity [158]. Similarly, another bispecific antibody (emicizumab, commercialized as Hemlibra®) mimics the activity of coagulation factor VIII by binding both coagulation factors IXa and X [159], two proteins that associate with lipid membranes through GLA domains [160]. In a recent presentation at the World Federation of Hemophilia World Congress (2018), data was presented from two phase III clinical trials. Hemophilia A patients treated with Hemlibra showed a pronounced (96%) decrease in the number of treated bleeds with no serious side effects. A bispecific antibody may also be used to 'staple' one surface protein to another one with a distinctive recycling pattern. The prolactin receptor is a protein with high internalization and degradation rate, whereas HER2 internalizes

but recycles back to the surface. By forcing the interaction between these two receptors, a bispecific antibody caused an increase in the internalization rate of HER2, an effect with potential use in antibody–drug conjugates [161].

7.4 Shuttling Across a Biological Barrier

Bispecific antibodies where one specificity is used to gain access to a tissue or cellular compartment and the second specificity binds its target to block or promote a process, has been referred to as a 'Trojan horse' strategy. The most extended application with this strategy is crossing the BBB (Table 4). Here, bispecific antibodies require one specificity binding to a receptor capable of mediating transcytosis, for example the transferrin (TfR) or insulin receptors, or carriers of metabolites such as the GLUT1 glucose transporter and LAT1 amino acid transporter [162]. By using this approach, the brain-to-blood IgG concentration ratio can be increased from the normal 0.1 to 2–3% [163]. The second specificity of the antibody is directed against a protein involved in a neurological disease such as Alzheimer's disease, Parkinson's disease, and brain tumors. A challenge for this application is the requirement of a binding affinity strong enough for the shuttling receptor to mediate efficient endocytosis, but not so strong that the antibody cannot be released in the brain parenchyma due to an altered TfR intracellular trafficking. This is usually achieved by modulating the affinity for TfR [164] and by using monovalent binding formats to TfR [165], the most extensively used shuttle. It is important to note that not only structurally monovalent formats can be used to this end, but also formats that are bivalent but can only engage the TfR monovalently, as described by Hultqvist et al. [166]. Shuttling across the BBB with bispecific antibodies targeting the TfR is an interesting case of the potential intricate relationship between the application, the effector function, and the antibody format. Some neurological applications of bispecific antibodies may benefit from effector competent molecules. For example, in Alzheimer's disease, removal of the aggregated β amyloid by FcγR-mediated engulfment by the microglia could be important for the therapeutic effect. However, it has been shown that a bispecific IgG antibody with effector function targeting the TfR led to an acute lethargy in mice due to the depletion of reticulocytes, immature red blood cells that express high levels of TfR [167].This effect could be prevented by using an effectorless molecule, suggesting that effector attenuated formats could be required for advancing bispecific antibodies using TfR safely in the clinic [167]. Interestingly, a recent publication using a bispecific antibody that also targets TfR but using an asymmetric IgG single-chain Fab fusion format (called Brain Shuttle mAb, BS-mAb), reported the molecule had no effector function in the periphery but it was capable of engaging with FcγR-bearing cells once in the central nervous system (CNS) compartment [109]. The hypothesis explaining this behavior is that when bound to TfR-expressing cells in the periphery, the orientation of the anti-TfR module at the C-terminus of the mAb leads to the presentation of the Fc in an inverted configuration with respect to the FcγR on adjacent effector cells. However, once in the brain parenchyma, binding to the target (β amyloid) results in the unobstructed presentation of the Fc to microglia cells [109]. This finding may help in designing bispecific antibodies with the required effector function capabilities to maximize efficacy and safety for neurological applications. In addition to bispecific antibodies, bifunctional molecules are also being developed to use the Trojan horse approach to cross the BBB. The enzyme replacement therapy (ERT) is a therapeutic approach for different pathologies related to the CNS [168, 169]. Hurler syndrome (or mucopolysaccharidosis I) is an inherited autosomal recessive lysosomal storage disorder caused by mutations in the gene encoding the lysosomal enzyme α-L-iduronidase (IDUA). AGT-181 is a bifunctional molecule developed by ArmaGen where the IDUA enzyme is fused to the C-terminus of the heavy chain of an antibody against the insulin receptor [170].The molecule was tested successfully in mice and monkeys [171, 172], and is currently in phase II clinical trials in pediatric patients (Table 3). Hunter syndrome is caused by a deficiency of the lysosomal enzyme iduronate-2-sulfatase. AGT-182 is another enzyme-mAb bifunctional molecule [173] that is in phase I clinical trials (Table 3). Although these are the two lysosomal storage diseases for which therapeutics are being tested in clinical trials, other bifunctional molecules are being tested preclinically for other indications [174]. The Trojan horse approach can be used to cross barriers other than the BBB. In a bispecific antibody developed to treat Ebola virus infection, one of the antibodies was selected to bind a structural protein on the surface of the virus, while the second specificity blocked entry of the virus by binding to a cryptic epitope only exposed after cleavage in the endosomes [175]. Unlike crossing the BBB, this application does not require a delicate balance between binding and release of one of the targets, and thus allows for more flexibility in terms of the formats that can be used. Given that many important functions happen in immune privileged and other compartments not usually accessible to antibodies, it is likely this strategy could be adopted to deliver antibodies to other localizations of therapeutic importance.

7.5 Hyperclustering of Membrane or Soluble Proteins

The formation of higher order complexes of membrane receptors is a phenomenon that can be exploited to improve the activity of antibody–drug conjugates or agonistic antibodies. Receptor crosslinking is a well known mechanism

to induce internalization and lysosomal degradation [212]; in one example, researchers in MedImmune developed a tetravalent scFv-IgG biparatopic anti-HER2 antibody that showed increased clustering formation and internalization potential than the parental antibodies (trastuzumab and 39S) [213]. With regard to the agonism applications, Brunker et al. from Roche developed a bispecific tetravalent CrossMAb against the death receptor 5 (DR5) and the fibroblast activating protein (FAP). The molecule induced FAP-binding-dependent hyperclustering and led to more potent apoptosis [214]. In the case of soluble proteins, bispecific antibodies can be used to promote the formation of higher complexes that are cleared from circulation more efficiently, as it has been shown using biparatopic antibodies against insulin-like growth factor 2 (IGF-2) [176] and interleukin 6 [177]. Although no formal comparison between formats has been published yet for this application, it is likely that formats that are multivalent for each specificity (as is the case of the two examples cited), with high flexibility in each binding domain, will bind multiple targets simultaneously and efficiently, leading to the formation of a high-order complex.

7.6 Cell- or Tissue-Specific Antibody Delivery or Activation

Most solid tumor targets are expressed in normal tissue to some extent, and if the differential of expression between tumor and normal tissue is not enough to support a positive therapeutic index, one potential strategy that is being explored is the simultaneous targeting of a second tumor-associated protein (Table 5). An example of this application was published by Gantke et al., where they constructed a trispecific antibody directed against B-cell maturation antigen (BCMA) and CD200, two B-cell targets, and CD16 to recruit NK cells. BCMA is an attractive antigen for multiple myeloma but low expression in keratinocytes has been reported, thus a dual targeting approach could increase selectivity towards double positive plasma cells. Results showed that indeed the trispecific antibody was significantly more potent towards cells expressing both antigens than any antigen alone [207].

Administration of a systemically active therapeutic may have adverse effects that could be mitigated by a more targeted delivery of the drug by virtue of a bispecific antibody. T-cell costimulatory molecules are targets pursued to boost the activity of T cells in cancer immunotherapy. Activation is usually triggered by the clustering of the receptor; however, activation outside of the tumor may have undesired side effects. For example, the use of agonistic mAbs against 4-1BB (CD137) [178, 179] and CD40 [180] may lead to hepatotoxicity. As a way to overcome the non-tumor activation, different bispecific molecules are being tested. ABBV-428 is a bispecific antibody targeting mesothelin and CD40 for the tumor-specific costimulation of T cells. Similarly, Roche is developing a bifunctional molecule combining the 4-1BB ligand with an anti-FAP antibody for the treatment of solid tumors [181]. Another example of a target that may benefit from a non-systemic blockade is TNF. Anti-TNFα antibodies are used for a variety of inflammatory conditions such as ulcerative colitis, Crohn's disease, rheumatoid arthritis, and severe chronic plaque psoriasis [182]. Systemic administration of anti-TNFα, however, increases the risk of opportunistic infections [183], including the reactivation of latent tuberculosis, and it may also cause autoimmune conditions [184]. TNF can be produced by stroma cells and different immune cells. It has been reported that the cell type producing TNF may dictate its specific functions in protective and autoreactive immune responses [185]. To selectively inhibit TNF produced by monocytes and macrophages, Efimov et al. used a bispecific antibody against TNF and F4/80, a cell-surface molecule highly expressed in those cells, resulting in a more efficient drug in a hepatotoxicity model [186, 187]. In addition to inhibiting soluble factors, bispecific antibodies can be used to inhibit cell surface receptors in a more tissue-specific fashion. Using the Wnt/β catenin pathways as a model, Lee et al. combined an antibody against the Wnt-associated protein LRP6 (the effector antigen) with different antibodies targeting different cell-type-associated proteins (the guide antigen) (ICAM-1, ALCAM, EphA2) [188]. The resulting antibodies showed an increased potency to inhibit Wnt signaling compared with the non-targeted anti-LRP6 antibody, but more importantly, inhibition was cell-type specific, as inhibition of signaling on cells not expressing the guide antigen was ~ 100-fold less potent [188]. Tumor growth factor β (TGF-β) is another protein with therapeutic value in oncology [189] and in renal fibrosis indications [190]. Interest in targeting TGF-β in oncology has been rising in light of reports showing inhibition of TGF-β enhances the action of checkpoint inhibitors [191]. Because TGF-β is a pleiotropic hormone, its chronic systemic inhibition may lead to multiple side effects, like compromised wound healing, tissue regeneration, and inflammatory function [192–194], and a more localized inhibition would be preferred. To explore this possibility in the context of kidney disease, McGaraughty et al. utilized a bispecific DVD antibody targeting TGF-β and the extra domain A isoform of fibronectin, which is overexpressed in the diseased kidney but absent in circulation [195, 196]. The bispecific molecule was shown to localize in the kidney and showed anti-fibrotic activity [197], lending support to the idea that this type of approach may be used for safer anti-TGF-β therapeutics as well as for other targets with multiple functions. In this example, the anti-fibronectin tissue arm has only the tissue delivery function. It is possible, however, that one arm has dual function like tissue delivery and inhibition of a pathway. M7824 is a bifunctional molecule

consisting of an anti-PDL-1 mAb fused to the extracellular domain of the TGF-β receptor II that acts as a trap for all three TGF-β isoforms. Expression of PDL-1 is increased in some tumors and the blockade of its interaction with PD-1 is an attractive therapeutic strategy [198]. Preclinical experiments showed M7824 had anti-tumor activity and although it produced a reduction of TGF-β levels in both the tumor microenvironment and in plasma [199], it is possible that by carefully engineering the affinities a more localized inhibition could be achieved.

In the examples described above, the tissue selectivity is conferred directly by the tissue-targeting arm. A more complex approach is combining improved tissue distribution with tissue activation, as described before for novel T-cell engager formats. In one example not related to T-cell engagers, Ferrari et al. exploited the fact that the inner domain in DVD antibodies sometimes shows a reduced binding affinity due to steric hindrance from the outer domain. They created a bispecific DVD IgG targeting the intercellular adhesion molecule 1 (ICAM1) (overexpressed in arthritic tissue) with the outer variable domain and TNF with the inner variable domain. The intact antibody had minimal TNF-binding activity due to the steric hindrance imparted by the outer variable domain. The linkers connecting the outer and inner variable domains (anti-TNF) contained a matrix metalloprotease 1 (MMP1) cleavage site, so upon accumulation in arthritic tissue, the linkers became cleaved and the anti-TNF unmasked [200]. Thus, bispecific antibodies can be carefully designed to support a more selective effector cell retargeting, hormone sequestration, receptor downregulation, or agonism.

The use of nanocarriers has been increasingly explored as an alternative modality for drug delivery. Minicells are particles derived from bacteria that can be loaded with different chemotherapeutics or miRNA being developed as therapeutics by EnGeneIC. Bispecific antibodies against the O-antigen component of the lipopolysaccharide (LPS) and various tumor-associated antigens were shown to effectively produce tumor regression in mice using minicells loaded with the chemotherapeutic doxorubicin [201]. Similarly loaded minicells targeted using an anti-LPS/EGFR were initially tested in dogs with spontaneous brain tumors. Results showed complete or > 90% tumor regression in 23% of the animals with no major clinical side effects [202]. Recently, a phase I clinical trial using paclitaxel-loaded minicells was completed, showing the treatment was well tolerated. Forty-five percent of the patients achieved stable disease but no objective responses were seen, presumably because the patients had acquired resistance to the drug [203]. The most advanced minicell-based therapeutic, currently in phase II, is one where the particles contain miR-16, a microRNA dysregulated in many cancer types. The minicells were tested in patients with malignant pleura mesothelioma with an acceptable safety profile and some signs of clinical activity [204]. The use of bispecific antibodies to direct other nanocarriers such as liposomes has also been described. A recent report described an anti-poly-ethylene glycol (PEG)/anti-EGFR bispecific antibody for the delivery of PEGylated nanomedicines to EGFR-expressing tumors [205]. Liposomes could be directly functionalized to incorporate antibodies directing them to the desired tissues, but some potential drawbacks to this approach have been raised [205]. Because it is common that liposomes are derivatized with PEG, the strategy could be applied to off-the-shelf drugs like the FDA-approved Doxil (PEGylated liposomes containing doxorubicin). Related to the use of bispecific antibodies for the delivery of particles, another application is found in the field of regenerative medicine. Particles conjugated to antibodies against stem cells and markers expressed by injured cells have been used to increase the efficiency of delivering the therapeutic cells [206].

8 Challenges Advancing Bispecific Antibodies to the Clinic

Significant progress has been made on engineering different solutions for the chain pairing problem in ways compatible with industrial production. This has allowed the progression of numerous molecules to clinical trials; however, in some cases this has not been without facing hurdles intrinsic to the bispecific nature of the antibodies.

8.1 More Challenging Safety Assessment Studies

Before a new drug can be tested in phase I trials, its toxicity in animals needs to be investigated to inform a safe starting dose in humans. One approach is to base the first-in-human dose on the no-observed adverse effect level (NOAEL) in a relevant species. A relevant species for a toxicity assessment should express the antigen with a similar tissue distribution and should bind the therapeutic candidate such that there is pharmacological activity. Because these requirements apply to each antibody specificity in the same species, finding a relevant animal model for toxicity studies could be more challenging for some bispecific (or multispecific) antibodies. If no cross-reactive species suitable for toxicity evaluation is identified, two alternative approaches are the use of surrogate cross-reactive molecules or the use of transgenic animal models expressing the human protein(s). These alternative routes, however, demand significant efforts to characterize the suitability of the models biochemically and functionally. The development of anti-carcinoembryonic antigen (CEA) T-cell bispecific antibody (TCB) is a recent example of the

exploration of different routes to evaluate preclinical toxicity [216]. While the anti-CD3 arm in the antibody cross-reacts with cynomolgus CD3, the anti-CEA antibody does not, ruling out the potential use of cynomolgus monkeys as a toxicity species with the clinical bispecific antibody. A surrogate antibody specific for cynomolgus CEA was tested instead, but functional in vitro studies showed that it did not reproduce the activity of the clinical candidate with human cells. As an alternative approach, a transgenic mouse model was created expressing both human CEA and CD3ε, but the expression of CEA did not show the same tissue distribution as in humans, nor did splenocytes from the transgenic animal show a comparable potency to human peripheral blood mononuclear cells (PBMCs) in cytotoxicity in vitro assays. In the absence of a relevant species to conduct toxicity studies, a minimum anticipated biological effect level (MABEL) approach was used to estimate the first-in-human dose. A series of functional in vitro assays (cell killing, T-cell activation, T-cell proliferation, cytokine release) was carried out and the EC_{20} of the most sensitive (cell killing) was used to derive the starting dose in patients. Other examples illustrating the challenges bispecific antibodies pose to preclinical safety assessment can be found in the review by Prell et al. [217]. In summary, although alternative approaches exist to calculate a safe first-in-human dose, bispecific antibodies may require a longer path demanding additional resources for the validation of the proper model.

8.2 Potentially Higher Immunogenicity

A different challenge that is also inheritably associated with more complex or heavily engineered molecules is the higher immunogenic potential. As additional specificities or functions are recruited onto antibodies, keeping the normal architecture of an IgG becomes more difficult, and divergences from the natural sequence increase the immunogenic potential. Given the fact that ADAs could potentially cause the failure of a therapeutic program, it becomes very useful to have tools to assess their immunogenicity before reaching clinical trials. Different methods are available to predict the immunogenicity of biotherapeutics, including in silico, in vitro and in vivo approaches. Although each of them has limitations when used individually, the integrated use of in silico and different in vitro assays are refining our ability to predict the immunogenicity of biotherapeutics. However, they are currently mainly used for assisting in candidate selection and not for making go/no go decisions [218]. Further improvement in the tool box of immunogenicity assays and a wider use of them could improve their validation and pave the way for reducing the number of bispecific antibodies, and biotherapeutics in general, that fail due to immunogenicity.

9 Conclusion and Future Perspectives

An indication of the success of bispecific antibodies is offered by a current market analysis that estimates the global market for bispecific antibodies will reach US$5.8 billion by 2024 (Bispecific Antibody Therapeutics Market [2nd Edition], 2014–2024). Blinatomomab, a bispecific antibody representative of the first envisioned application, the recruitment of T cells, has already received approval for commercialization and a swelling number of molecules with this mechanism of action are entering clinical trials. In addition to this well validated modality, novel, more experimental uses of bispecific antibodies keep emerging. What makes bispecific antibodies so attractive and useful is that they open spaces previously not accessible to therapeutic antibodies, extending their reach and making them more efficacious and potentially safer. The improved safety is a possibility that still needs to be formally shown. However, the preclinical examples of bispecific antibodies producing a more selective tissue delivery supports the notion that bispecific antibodies could open the doors for safer therapeutics in a range of applications. As a token of the growing interest in this area it is worth mentioning that Pandion Therapeutics, a recently (2017) founded company, has made localized immunomodulation with bispecific antibodies the main focus of their strategy for treating autoimmune and inflammation-related conditions.

The need to endow antibodies with additional functions is driving the generation of more complex molecules. In addition to multi-specific/multivalent antibodies, an area of growing interest is the combination of antibodies and ligands or enzymes to create bi- or multifunctional molecules. Ligands are used in some cases because their function cannot be mimicked with the same efficiency by antibodies, or because they provide catalytic activity in the case of enzymes. Examples of antibodies delivering cytokines include IL15 fused to fragments [145] or to an Fc-containing molecule [219] (a more comprehensive list is available here [220]). Bifunctional antibodies are also used to deliver ligands to costimulatory molecules like 4-1BB [181], to deliver enzymes for neurological applications [221], or for sequestering a tumorigenic hormone in the tumor microenvironment [222, 223]. A challenge inherently associated with more complex or heavily engineered molecules is the higher immunogenic potential. Complete data sets from clinical trials with different antibody formats are still needed to have a clear assessment of the actual liabilities. However, given the large number of bispecific antibodies currently in clinical trials, it is likely the body of information will increase in the next few years. The resulting data will be extremely useful to inform on the efficacy and immunogenicity of the different formats

and perhaps guide future engineering efforts to overcome their liabilities.

In summary, bispecific antibodies have matured as a real therapeutic option for many applications and they are positioned to continue to expand the frontiers of antibody therapeutics.

Compliance with Ethical Standards

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