Review Article

Biology drives the discovery of bispecific antibodies as innovative therapeutics

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ABSTRACT

A bispecific antibody (bsAb) is able to bind two different targets or two distinct epitopes on the same target. Broadly speaking, bsAbs can include any single molecule entity containing dual specificities with at least one being antigen-binding antibody domain. Besides additive effect or synergistic effect, the most fascinating applications of bsAbs are to enable novel and often therapeutically important concepts otherwise impossible by using monoclonal antibodies alone or their combination. This so-called obligate bsAbs could open up completely new avenue for developing novel therapeutics. With evolving understanding of structural architecture of various natural or engineered antigen-binding immunoglobulin domains and the connection of different domains of an immunoglobulin molecule, and with greatly improved understanding of molecular mechanisms of many biological processes, the landscape of therapeutic bsAbs has significantly changed in recent years. As of September 2019, over 110 bsAbs are under active clinical development, and near 180 in preclinical development. In this review article, we introduce a system that classifies bsAb formats into 30 categories based on their antigen-binding domains and the presence or absence of Fc domain. We further review the biology applications of approximately 290 bsAbs currently in preclinical and clinical development, with the attempt to illustrate the principle of selecting a bispecific format to meet biology needs and selecting a bispecific molecule as a clinical development candidate by 6 critical criteria. Given the novel mechanisms of many bsAbs, the potential unknown safety risk and risk/benefit should be evaluated carefully during preclinical and clinical development stages. Nevertheless we are optimistic that next decade will witness clinical success of bsAbs or multispecific antibodies employing some novel mechanisms of action and deliver the promise as next wave of antibody-based therapeutics.

Statement of Significance: This article comprehensively reviewed various bispecific antibody formats and the biology driving the design and selection of a right bispecific antibody to enable novel therapeutic concept and match intended therapeutic applications. The principles and the examples discussed could provide a general guidance for people interested in exploring bispecific antibody therapeutics.

KEYWORDS: bispecific antibody; bsAb; multispecific antibody; msAb

A BRIEF HISTORICAL VIEW OF BISPECIFIC ANTIBODIES

The invention of hybridoma technology in 1975 marked the arrival of new era of monoclonal antibody (mAb)-based therapy [1]. However, the first wave of clinical attempts with mouse antihuman mAb therapeutics during 1975–86 largely failed, due to immunogenicity of mouse sequences, with only one mAb (anti-CD3 muromonab) being approved. It took another decade for the field to solve the immunogenicity issues, and the lessons learned from the first wave of clinical trials of antibody therapeutics is the key driver leading to invention of innovative antibody humanization technologies represented by antibody chimerization, CDR graft, in vitro display of human...

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antibody repertoire, and human immunoglobulin transgenic rodents. The approval of rituximab by the US FDA in 1997 marked the field entering into the booming stage. About 100 antibody-based therapeutics have been approved by the regulatory agencies worldwide, since then, antibody therapeutics have now become one of the mainstays for developing new medicines. The history of development of bispecific antibodies (bsAbs) almost followed the footprint of the development of mAb therapeutics. As illustrated in a recent review article [2], starting in the 1960s, scientists explored generation of antigen-binding fragments (Fab) from two different polyclonal sera and reassociated them into bispecific F(ab′)2 molecules. After hybridoma technology was established in 1975, chemical conjugation of two rodent mAbs or fusion of two antibody-producing hybridomas (so-called quodroma) was explored immediately to make bsAbs with defined specificities. The first therapeutic bsAb catumaxomab (Removab®) approved by the EMA in 2009 was made by this early technology. The bsAbs made by these two methods prior to establishing antibody humanization technologies, however, suffered from the same issue of immunogenicity in addition to stability, solubility, and manufacturability challenges. The development of methods to produce recombinant antibodies in the 1980s enabled the rapid generation of various bsAbs with defined structure, composition, and biochemical, functional, and pharmacological properties, but it still took scientists more than 2 decades to really understand the unique structural features of various antigen-binding building blocks such as Fab, Fv, scFv, SDA, etc., to develop various innovative engineering solutions to generate homo- and heterodimerization building blocks necessary for making various bispecific formats and most importantly understand the structural biology of how to connect them together to enable various biology concepts while maintaining favorable developability. In the later paragraphs, we will review the evolution of some of those landmark solutions for bsAb construction. But before we get into detailed discussion of how to make various recombinant bsAbs, we will discuss the principles governing how to define and identify a good bsAb therapeutics first.

THE PRINCIPLES GOVERNING A GOOD THERAPEUTIC BISPECIFIC ANTIBODY

Though mAbs have demonstrated definitive therapeutic benefits in multiple disease areas, it is believed that bsAbs can further advance the success of therapeutic antibodies by enabling the molecules with new mechanisms of action (MOAs) and by providing new functional advantages that cannot be achieved by mAbs. We believe that identification of a good bsAb should be based on three principles (Fig. 1): (1) the molecule should be able to provide unique biological function to achieve desired efficacy with appropriate safety profile, driven by unique biology; (2) the format chosen should enable the molecule to fulfill its proposed function, match biology with an optimal format; and (3) the molecule selected as a clinical development candidate should satisfy the six criteria critical for clinical development and commercial manufacturing, i.e., desired clinical efficacy, appropriate safety profile, favorable pharmacokinetic/pharmacodynamic (PK/PD) properties, appropriate physicochemical properties, scalable manufacturability, and minimal or no immunogenicity risk—select a right molecule. Unfortunately, these six criteria, particularly those critical for biological function (efficacy, safety, PK/PD, immunogenicity) and those critical for developability (expression, homogeneity, solubility, stability, viscosity, formulation ability, etc.), often are not correlated with each other, sometimes even counterbalance each other that requires balancing when selecting a therapeutic molecule. Identification of a good therapeutic bispecific molecule therefore usually requires starting with good therapeutic molecular design defined by molecular product profile (MPP) that is developed based on target product profile (TPP), followed by rigorous molecular and functional screen, selection and characterization using pharmacological assays, mechanistic and/or disease models, and other preclinical translational systems relevant to the human disease one intends to treat.

THE MAKING OF RECOMBINANT BISPECIFIC ANTIBODIES

In a recent review article, Brinkmann and Kontermann thoroughly reviewed many experimentally verified formats that had been described in the literature as of September 2016 [3]. We concur with their opinion that besides the freedom-to-operate (FTO) and the desire to generate proprietary intellectual properties (IP) for competitive reason, one of the critical drivers for explosive diversity of so many bsAb formats is the plethora of desired functionalities and applications of bsAbs. Format variability is essential to serve diverse bsAb applications defined by different TPP. These formats may vary in size, domain composition and arrangement, binding kinetics and valencies, flexibility and geometry of their binding modules, as well as in their biodistribution and pharmacokinetic properties to fulfill a particular clinical application. Small variations, such as minor changes in linker length or composition of domains, can be crucial determinants for functionality. Some designed parameters may be deduced from structural modeling of drug-target interaction. In many cases, however, a suitable molecule must be identified by generating and comparing the functionalities of different formats and different molecules in the systems relevant to clinical settings.

Here we review various bsAb formats and classify them into 30 categories: (1) what are the building blocks of antigen-binding and their combination, and (2) whether they contain fragment of crystallizable region (Fc) domain. From published reports and our practice, most bispecific formats contain the antigen-binding sites derived from immunoglobulin domain of native antibodies. We identify single-domain antibody (SDA or VHH), variable fragment (Fv), single-chain variable fragment (scFv), Fab, and single-chain antigen-binding fragment (scFab) as the five key building blocks of bispecific formats. As shown in Fig. 2, most of bsAb formats can be classified into 30 groups based on the above classification. As there are more than 200
bispecific formats from published data and our practice, we do not intend to list all these formats in Fig. 2. Instead, we have just listed an example of each category to illustrate the concept.

In each category, the bispecific formats can be further classified by their geometry (such as homodimer vs. heterodimer) and valency (number of antigen-binding sites). A bsAb with one binding site to target A and one binding site to target B is called $1_+1$ format. Similarly there are $1_+2$, $1_+3$, and $2_+2$ formats. The formats with more than four antigen-binding sites are uncommon but growing, so they are just mentioned as examples in this review.

In addition to the building blocks, absence or presence of Fc, and different valency, multiple fusion sites of Fc-containing formats increase the complexity of bispecific formats. As shown in Fig. 3A, an antigen-binding building block can be fused to N-terminus or C-terminus of an Fc fragment or inserted between CH2 domain and CH3 domain. On a heterodimeric Fc-containing bispecific format, there are at least six fusion sites. If an Fc-containing format also comprises of CL, the fusion sites increase to 12 (Fig. 3A). Moreover, theoretically all the loops of each immunoglobulin domain (CL, CH1, CH2, and CH3) can be used as fusion sites to integrate an antigen-binding building block. It becomes obvious to employ these fusion sites to make a bispecific format with desired binding activity.

Bispecific molecules containing non-antibody-binding domains such as peptides, ligands, receptors, or alternative scaffolds may not fall into this classification system. However, depending on how many polypeptide chains of the antigen-binding sites are used, the non-antibody bispecific molecules can be constructed using similar approaches as the above bsAbs.

**Bispecific antibody fragments without Fc**

In this category, all antigen-binding sites are from the aforementioned building blocks (SDA, Fv, scFv, Fab, and scFab) and the bsAbs do not contain Fc. Many different bispecific formats, including $1_+1$, $1_+2$, $1_+3$, and $2_+2$ formats, and trispecific formats have been used for preclinical and clinical development (Tables 1–6). BsAb fragments usually are smaller than IgG and lack of Fc-related functions such as FcγR-, FcRn-, and complement-binding and related activities. Due to large number of the bsAb fragment formats, only some examples of bsAbs fragments are briefly described below.
Table 1. Programs in clinical and preclinical stages to block the angiogenesis and/or tumorigenesis for cancer treatment

| Antibody name      | Organization                                      | Targets                   | Highest phase | Biological function     | Type of mechanism                  | Format                        | Clinical studies               |
|--------------------|---------------------------------------------------|---------------------------|---------------|--------------------------|------------------------------------|-------------------------------|-------------------------------|
| Dilpacinab, ABT-165| AbbVie                                            | VEGF × DLL4               | Phase II      | Anti-angiogenesis        | Combinatorial effect                | Fab + Fv with Fc, 2 + 2       | NCT01946074, NCT01944426, NCT03368859, NCT03418532 |
| MP0250             | Molecular Partners AG                             | VEGF × HGF × albumin      | Phase II      | Anti-angiogenesis        | Combinatorial effect                | Scaffold 1 + 1 + 1             | NCT02194426, NCT03136653, NCT03418532 |
| ABL-001, NOV-1501, TR-009 Vanucizumab, RG-7221 | ABL Bio, TRIGR Therapeutics, Roche, Harvard Medical School, National Cancer Centre of Singapore | ANGPT2 × VEGF            | Phase I       | Anti-angiogenesis        | Combinatorial effect                | Fab + scFv with Fc, 2 + 2     | NCT01688206, NCT02141295, NCT0265416 |
| BI-836880          | Boehringer Ingelheim, Sanofi                      | ANGPT2 × VEGF, albumin    | Phase I       | Anti-angiogenesis        | Combinatorial effect                | VH + VH, 1 + 1 + 1             | NCT02674152, NCT02689505, NCT03468426, NCT03861234, NCT03972150 |
| Navicixizumab, OMP-305BB83 | OncoMed Pharmaceuticals                          | VEGF × DLL4               | Phase I       | Anti-angiogenesis        | Combinatorial effect                | Fab + Fab with Fc, 1 + 1       | NCT02298387, NCT03030287, NCT03035253 |
| KN-026             | Jiangsu Alphamab Biopharmaceuticals              | HER2 × HER2               | Phase II      | Anti-tumorigenesis       | Biparatopic                         | Fab + Fab with Fc, 1 + 1       | NCT03619681, NCT03847168, NCT03925974, NCT04040699 |
| ZW-25              | Zymeworks, BeiGene                                 | HER2 × HER2               | Phase II      | Anti-tumorigenesis       | Biparatopic                         | Fab + scFv with Fc, 1 + 1      | NCT02892123, NCT03929666, NCT02912494 |
| MCLA-128           | Merus                                             | HER3 × HER2               | Phase II      | Anti-tumorigenesis       | Combinatorial effect                | Fab + Fab with Fc, 1 + 1       | NCT03321981, NCT03797391 |
| EMB-01, FIT-013a   | EpimAb Biotherapeutics                            | EGFR × cMET               | Phase I/II     | Anti-tumorigenesis       | Combinatorial effect                | Fab + Fab with Fc, 2 + 2       | NCT02609776, NCT04077463, NCT03912441 |
| JNJ-61186372, JNJ-6372 BCD-147 | Janssen                                         | EGFR × cMET               | Phase I       | Anti-tumorigenesis       | Combinatorial effect                | Fab + scFv with Fc, 1 + 2      | NCT03842085 |
| MBS-301            | Beijing Mabworks Biotech                          | HER2 × HER2               | Phase I       | Anti-tumorigenesis       | Biparatopic                         | Fab + Fab with Fc, 1 + 1       | NCT03842085 |

Continued
| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|------------------|
| BI-905677    | Boehringer Ingelheim | LRP5/6 | Phase I        | Anti-tumorigenesis  | Biparatopic       | SDA + SDA, 1 + 1 | NCT03604445 |
| MP0274       | Molecular Partners AG | Her2 × Her2 | Phase I | Anti-tumorigenesis  | Biparatopic       | SCAFFOLD, 1 + 1 | NCT03084926 |
| VEGFR2/Ang2  | Eli Lilly & Co | VEGFR2 × ANGPT2 | Preclinical | Anti-angiogenesis   | Combinatorial effect | Fab + scFv with Fc, 2 + 2 | NA |
| FS-101       | F-star Therapeutics Ltd | EGFR × HGF | Preclinical | Anti-angiogenesis   | Combinatorial effect | Fab + SDA with Fc, 2 + 2 | NA |
| MP-E-8-3/1959 | MediaPharma | Endosialin × LGALS3BP | Preclinical | Anti-angiogenesis   | Combinatorial effect | Not disclosed | NA |
| PMC-001      | PharmAbcine | Tie-2 × VEGFR2 | Preclinical | Anti-angiogenesis   | Combinatorial effect | Fab + LIGAND with Fc, 2 + 2 | NA |
| PMC-201      | PharmAbcine | DLL4 × VEGFR2 | Preclinical | Anti-angiogenesis   | Combinatorial effect | Not disclosed | NA |
| PMC-404      | PharmAbcine | ANGPT2 × VEGF-c | Preclinical | Anti-angiogenesis   | Combinatorial effect | Not disclosed | NA |
| MCLA-129     | Betta Pharmaceuticals; Merus | VEGF × cMET | Preclinical | Anti-angiogenesis, anti-tumorigenesis | Combinatorial effect | Fab + Fab with Fc, 1 + 1 | NA |
| MP-EV20/1959 | MediaPharma | HER3 × LGALS3BP | Preclinical | Anti-angiogenesis, anti-tumorigenesis | Combinatorial effect | Not disclosed | NA |
| CKD-702      | Chong Kun Dang Pharmaceutical | EGFR × cMET | Preclinical | Anti-tumorigenesis  | Combinatorial effect | Fab + scFv with Fc, 2 + 2 | NA |
| CBA-0702     | Sorrento Therapeutics | Her3 × cMET | Preclinical | Anti-tumorigenesis  | Combinatorial effect | scFv + scFv with Fc, 1 + 1 | NA |
| SRB-19       | SunRock Biopharma; Biocad Ltd | EGFR × Her3 | Preclinical | Anti-tumorigenesis  | Combinatorial effect | Not disclosed | NA |
| BTA-106      | Zenyaku Kogyo Co Ltd | IgM × HLA-DR | Preclinical | Anti-tumorigenesis  | Combinatorial effect | Fab + Fab with Fc, 1 + 1 | NA |
| TXB4-BC2     | Ossianix Inc | TiR × EGFRvIII | Preclinical | Anti-tumorigenesis  | Trojan horse | Fab + SDA with Fc, 2 + 2 | NA |
| TXB4-BC1     | Ossianix Inc | TiR × CD20 | Preclinical | Anti-tumorigenesis  | Trojan horse | Fab + SDA with Fc, 2 + 2 | NA |
| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|------------------|
| KN-046        | Jiangsu Alphamab Biopharmaceuticals | PD-L1 × CTLA-4 | Phase II | Enhance tumor immunity | Tumor or tissue localization | SDA + SDA with Fc, 2 + 2 | NCT03529526, NCT03733951, NCT03883848, NCT03872791, NCT03925870, NCT03927495, NCT04040699 |
| AK-104        | Akeso Biopharma | PD-1 × CTLA-4 | Phase I/II | Enhance tumor immunity | Combinatorial effect | Fab + scFv with Fc, 2 + 2 | NCT03261011, NCT03852251 |
| DuoBody-PD-L1x4-1BB, GEN-1046 | BioNTech, Genmab | PD-L1 × 4-1BB | Phase I/II | Enhance tumor immunity | Tumor or tissue localization | Fab + Fab with Fc, 1 + 1 | NCT03917381 |
| REGN-5678     | Regeneron    | PSMA × CD28  | Phase I      | Enhance tumor immunity | Tumor or tissue localization | Fab + Fab with Fc, 1 + 1 | NCT03972657 |
| FS118 mAb2, FS-118, LAG-3/PD-L1 mAb2 IBI-318 | F-star | PD-L1 × LAG-3 | Phase I | Enhance tumor immunity | Tumor or tissue localization | Fab + SDA, 2 + 2 | NCT03440437 |
|               | Innovoent Biologics, Lilly | PD-1 × PD-L1 | Phase I | Enhance tumor immunity | Promote downregulation | Not disclosed | NCT03875157 |
|               | Eli Lilly     | PD-1 × PD-L1 | Phase I | Enhance tumor immunity | Promote downregulation | Fab + Fab with Fc, 1 + 1 | NCT03936959 |
| MGD-013       | MacroGenics, ZAI Lab | PD-1 × LAG-3 | Phase I | Enhance tumor immunity | Combinatorial effect | Fv + Fv with Fc, 2 + 2 | NCT01070972, NCT0082364 |
| XmAb-23104    | Xencor       | PD-1 × ICOS  | Phase I | Enhance tumor immunity | Combinatorial effect | Fab + scFv with Fc, 1 + 1 | NCT03752398 |
| ABBV-428      | AbbVie       | MSLN × CD40  | Phase I | Enhance tumor immunity | Tumor or tissue localization | scFv + scFv with Fc, 2 + 2 | NCT02955251 |
| ADC-1015, ATOR-1015 | Alligator Bioscience | OX40 × CTLA-4 | Phase I | Enhance tumor immunity | Combinatorial effect | Fab + LIGAND with Fc, 2 + 2 | NCT03782467 |
| INBRX-105-1, INBRX-105, ES-101 MCLA-145 | Inhibrix, Elpiscience Biopharma, Incyte | PD-L1 × 4-1BB | Phase I | Enhance tumor immunity | Tumor or tissue localization | SDA + SDA with Fc, 2 + 2 | NCT03809624, NCT03877899, NCT03878279 |
| MEDI-5752     | MedImmune    | PD-1 × CTLA-4 | Phase I | Enhance tumor immunity | Combinatorial effect | Fab + Fab with Fc, 1 + 1 | NCT03922204 |
| MGD-019       | MacroGenics  | PD-1 × CTLA-4 | Phase I | Enhance tumor immunity | Combinatorial effect | Fv + Fv with Fc, 2 + 2 | NCT03530397 |
| PRS-343       | Pieris       | HER2 × 4-1BB | Phase I | Enhance tumor immunity | Tumor or tissue localization | Fab + SCAFFOLD with Fc, 2 + 2 | NCT03330561, NCT03650348 |
| RG-7769, RO-7121661 | Roche | PD-1 × TIM-3 | Phase I | Enhance tumor immunity | Combinatorial effect | Fab + Fab with Fc, 1 + 1 | NCT03708328 |

Continued
| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|------------------|
| XmAb-20717    | Xencor       | PD-1 × CTLA-4 | Phase I        | Enhance tumor immunity | Combinatorial effect | Fab + scFv with Fc, 1 + 1 | NCT03517488 |
| XmAb-22841    | Xencor       | CTLA-4 × LAG-3 | Phase I        | Enhance tumor immunity | Combinatorial effect | Fab + scFv with Fc, 1 + 1 | NCT03849469 |
| RG-7827       | Roche        | FAP × 4-1BB   | Phase I        | Enhance tumor immunity | Tumor or tissue localization | Fab + LIGAND with Fc, 1 + 3 | Company development pipeline |
| MP0310        | Molecular Partners AG, Amgen | FAP × CD40 | Phase I        | Enhance tumor immunity | Tumor or tissue localization | SCAFFOLD, 1 + 1 | NCT04049903 |
| HX-009        | HanX Biopharmaceuticals | PD-1 × CD47 | IND Filed | Enhance tumor immunity | Combinatorial effect | Fab + LIGAND with Fc, 2 + 2 | NCT04097769 |
| AK-112        | Akeso Biopharma | VEGF × PD-1 | IND Filed | Enhanced tumor immunity, anti-angiogenesis | Combinatorial effect | Fab + scFv with Fc, 2 + 2 | NCT04047290 |
| INV-531       | Invenra Inc  | OX40 biparatopic | Preclinical | Enhance tumor immunity | Biparotopic | Fab + Fab with Fc, 1 + 2 | NA |
| ATOR-1144     | Alligator Bioscience | GITR × CTLA-4 | Preclinical | Enhance tumor immunity | Combinatorial effect | Fab + LIGAND with Fc, 2 + 2 | NA |
| BH-2996 h     | Beijing Hanmi Pharmaceutical | PD-1 × PD-L1 | Preclinical | Enhance tumor immunity | Promote downregulation | Fab + Fab with Fc, 1 + 1 | NA |
| GEN-1042      | BioNTech; Genmab | CD40 × 4-1BB | Preclinical | Enhance tumor immunity | Combinatorial effect | Fab + Fab with Fc, 1 + 1 | NA |
| CB-213        | Crescendo Biologics | PD-1 × LAG-3 × albumin | Preclinical | Enhance tumor immunity | Combinatorial effect | SDA + SDA + SDA, 1 + 1 + 2 | NA |
| FS-120        | F-star Therapeutics | OX40 × 4-1BB | Preclinical | Enhance tumor immunity | Combinatorial effect | Fab + SDA, 2 + 2 | NA |
| MEDI-3387     | MedImmune LLC | GITR × PD-1 | Preclinical | Enhance tumor immunity | Combinatorial effect | Fab + LIGAND with Fc, 2 + 2 | NA |
| MEDI-5771     | MedImmune LLC | GITR × PD-1 | Preclinical | Enhance tumor immunity | Combinatorial effect | Fab + LIGAND with Fc, 2 + 2 | NA |
| PT-302        | Phanes Therapeutics | LAG-3 × TIM-3 | Preclinical | Enhance tumor immunity | Combinatorial effect | Not disclosed | NA |
| TSR-075       | TESARO Inc   | PD-1 × LAG-3 | Preclinical | Enhance tumor immunity | Combinatorial effect | Not disclosed | NA |
| PD-1/LAG-3 bispecific mAbs | Xencor Inc | PD-1 × LAG-3 | Preclinical | Enhance tumor immunity | Combinatorial effect | Fab + scFv with Fc, 1 + 1 | NA |
| AM-105        | AbClon Inc   | EGFR × 4-1BB | Preclinical | Enhance tumor immunity | Tumor or tissue localization | Not disclosed | NA |

Continued
| Antibody name       | Organization                                      | Targets                          | Highest phase | Biological function                              | Type of mechanism                                      | Format                  | Clinical studies |
|---------------------|---------------------------------------------------|----------------------------------|---------------|-------------------------------------------------|--------------------------------------------------------|-------------------------|------------------|
| ALG-APV-527         | Alligator; Aptevo Therapeutics Inc                | 5T4 × 4-1BB                     | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | scFv + scFv with Fc, 2 + 2 | NA               |
| BY-24.3             | Beijing Beyond; Hangzhou Sumgen                   | VEGF × PD-1                     | Preclinical   | Enhance tumor immunity                          | Combinatorial effect                                  | Not disclosed           | NA               |
| BH-2922             | Beijing Hanmi                                      | EGFR × PD-1                     | Preclinical   | Enhance tumor immunity                          | Combinatorial effect                                  | Fab + Fab with Fc, 1 + 1  | NA               |
| BH-2950             | Beijing Hanmi; Innoven                            | Her2 × PD-1                     | Preclinical   | Enhance tumor immunity                          | Combinatorial effect                                  | Fab + Fab with Fc, 1 + 1  | NA               |
| DuoBody-PD-L1x4-1BB | BioNTech; Genmab                                    | PD-L1 × 4-1BB                   | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | Fab + scFv with Fc, 2 + 2 | NA               |
| CDX-527             | Celldex Therapeutics                               | PD-L1 × CD27                    | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | SDA + SDA + SDA, 1 + 1 + 1 | NA               |
| CB-307              | Crescendo Biologics                                | PSMA × 4-1BB × albumin           | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | scFv + SDA + SDA, 1 + 1 + 1 | NA               |
| ND-021              | CStone; Numab                                       | PD-L1 × 4-1BB × albumin          | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | Fab + SDA, 2 + 2         | NA               |
| FS-222              | F-star                                            | PD-L1 × 4-1BB                   | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | Fab + SDA, 2 + 2         | NA               |
| EGF/CTLA-4 bispecific mAb2 | F-star                                      | EGFR × CTLA-4                   | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | Not disclosed            | NA               |
| IBI-323             | Innovent Biologics                                 | PD-L1 × LAG-3                    | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | Fab + SDA with Fc, 2 + 2  | NA               |
| KY-1055             | Kymab                                             | PD-L1 × ICOS                     | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | scFv + SDA, 3 + 3        | NA               |
| 1D8N/CEGa1          | LeadArtis                                         | EGFR × 4-1BB                    | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | Fab + Fv with Fc, 1 + 2  | NA               |
| 4-1BBx5T4           | MacroGenics                                        | 5T4 × 4-1BB                     | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | Fab + Fv with Fc, 1 + 2  | NA               |
| 4-1BBxHER2          | MacroGenics                                        | Her2 × 4-1BB                    | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | Fab + Fv with Fc, 1 + 2  | NA               |
| PD-L1x4-1BB         | MacroGenics Inc                                    | PD-L1 × 4-1BB                   | Preclinical   | Enhance tumor immunity                          | Tumor or tissue localization                           | Fab + Fv with Fc, 2 + 2  | NA               |

Continued
### Table 2. Continued

| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|-----------------|
| MEDI-1109     | MedImmune    | PD-L1 × OX40 | Preclinical   | Enhance tumor immunity | Tumor or tissue localization | Fab + LIGAND with Fc, 2 + 2 | NA |
| PRS-300 series A | Pieris     | Her2 × CTLA-4 | Preclinical   | Enhance tumor immunity | Tumor or tissue localization | Not disclosed | NA |
| PRS-342       | Pieris       | GPC3 × 4-1BB | Preclinical   | Enhance tumor immunity | Tumor or tissue localization | SCAFFOLD + SCAFFOLD with Fc, 2 + 2 | NA |
| PRS-344       | Pieris; Servier | PD-L1 × 4-1BB | Preclinical | Enhance tumor immunity | Tumor or tissue localization | Fab + SCAFFOLD with Fc, 2 + 2 | NA |
| PD-1 × BTLA   | Xencor       | BTLA × PD-1 | Preclinical   | Enhance tumor immunity | Combinatorial effect | Fab + scFv with Fc, 1 + 1 | NA |
| TXB4-BC3      | Ossianix Inc | TLR × PD-L1 | Preclinical   | Enhance tumor immunity | Trojan horse | Fab + SDA with Fc, 2 + 2 | NA |
| CBA-0710      | Sorrento     | cMET × PD-L1 | Preclinical   | Enhance tumor immunity, anti-tumorigenesis | Combinatorial effect | Fab + Fab with Fc, 1 + 1 | NA |

### Table 3. Programs in clinical and preclinical stages to modulate TME for cancer treatment

| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|-----------------|
| Bintrafusp alfa | GxaxoSmithKline, Merck KGaA | PD-L1 × TGFbeta | Phase III | Modulate TME | Tumor or tissue localization | Fab + RECEPTOR with Fc, 2 + 2 | NCT04066491, NCT03840902, NCT03833661, NCT03631706, NCT03840915, NCT02699515, NCT02517398 |
| AGEN-1423, GS-1423 | Agenus, Gilead | CD73 × TGFbeta | Phase I | Modulate TME | Combinatorial effect | Not disclosed | NCT03710265, NCT03774979 |
| SHR-1701      | Jiangsu Hengrui | PD-L1 × TGFbeta | Phase I | Modulate TME | Tumor or tissue localization | Fab + RECEPTOR with Fc, 2 + 2 | NCT03954704 |
| AK-123        | Akeso Biopharma | PD-1 × CD73 | Preclinical | Enhance tumor immunity, modulate TME | Tumor or tissue localization | Not disclosed | NA |
| UniTI-101     | Elstar Therapeutics | CCR2 × CSF1R | Preclinical | Modulate TME | Combinatorial effect | Fab + Fab with Fc, 1 + 1 | NA |
| FmAb-2        | Biocon; IATRICa | EGFR × TGFbeta | Preclinical | Modulate TME | Tumor or tissue localization | Fab + RECEPTOR with Fc, 2 + 2 | NA |
| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|-----------------|
| Tebentafusp   | Immunocore   | gp100/HLA-A\(^*\)0201 × CD3 | Phase III | Target cell depletion | Cytotoxic effector engagement | TCR + scFv, 1 + 1 | NCT03070392, NCT02889861, NCT02570308, NCT02535078, NCT01211262, NCT01209676, NCT00889408, NCT02370160, NCT01221571, NCT02321592, NCT02665650, NCT03192202, NCT04074746 |
| OXS-1550, DT-2219 | GT Biopharma | CD19 × CD22 | Phase II | Target cell depletion | ADC | scFv + scFv, 1 + 1 | |
| AFM-13        | Affimed      | CD16 × CD30 | Phase II | Target cell depletion | Cytotoxic effector engagement | Fv + Fv, 2 + 2 | |
| Odronextamab, REGN-1979 | Regeneron | CD3 × CD20 | Phase II | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1 | NCT02651662, NCT03888105 |
| IMC-C103C     | Genentech; Immunocore | MAGE-A4/HLA-A0201 × CD3 | Phase II | Target cell depletion | Cytotoxic effector engagement | TCR + scFv, 1 + 1 | NCT03973333 |
| IMCnyeso      | GlaxoSmithKline; Immunocore | NY-ESO-1/HLA-A0201 × CD3 | Phase II | Target cell depletion | Cytotoxic effector engagement | TCR + scFv, 1 + 1 | NCT03515551 |
| Mosunetuzumab, RG-7828 | Genentech, Roche, Chugai | CD3 × CD20 | Phase I/II | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1 | NCT02500407, NCT03671018, NCT03677141, NCT03677154 |
| OXS-3550, CD161533 TriKE | GT Biopharma, Altor BioScience, U. Minnesota | CD16 × CD33, IL-15 | Phase I/II | Target cell depletion | Cytotoxic effector engagement | scFv + scFv + LIGAND, 1 + 1 + 1 | NCT03214666 |
| GEN-3013      | Genmab       | CD3 × CD20 | Phase I/II | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1 | NCT03625037 |
| MCLA-117      | Merus        | CD3 × CLEC12 | Phase I/II | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1 | NCT03038230 |
| Flotetuzumab, MGD-006, MGD-007 | MacroGenics, Servier | CD3 × CD123 | Phase I/II | Target cell depletion | Cytotoxic effector engagement | Fv + Fv, 1 + 1 | NCT02152956, NCT03739606, NCT02248805, NCT03531632, NCT03564340 |
| Cibisatamab, RO-6958688, RG-7802 | Genentech, Roche, Chugai | CD3 × CEA | Phase I/II | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1 | NCT02324257, NCT02650713, NCT03337698, NCT03866239 |

Continued
Table 4. Antibody Therapeutics, 2020

| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|------------------|--------|-----------------|
| huGD2-BsAb    | Y-mAbs       | CD3 × GD2 | Phase I/II     | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 2 + 2 | NA    |
| AMG-701       | Amgen        | CD3 × BCMA | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT03287908 |
| A-337         | Generon (Shanghai) | CD3 × EpCAM | Phase I        | Target cell depletion | Cytotoxic effector engagement | Fab + scFv, 1 + 2 | Company development pipeline |
| AMG-160       | Amgen        | CD3 × PSMA | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT03792841 |
| AMG-330, MT-114 | Amgen     | CD3 × CD33 | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT02520427 |
| AMG-424       | Amgen        | CD3 × CD38 | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT03445663 |
| AMG-427       | Amgen        | CD3 × FLT3 | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT03541369 |
| AMG-562       | Amgen        | CD3 × CD19 | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT03571828 |
| AMG-596       | Amgen        | CD3 × EGFRvIII | Phase I      | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT03296696 |
| AMG-673       | Amgen        | CD3 × CD33 | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT03224819 |
| AMG-757       | Amgen        | CD3 × DLL3 | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT03319940 |
| AMV-564, TandAb T564, APVO-436 | Affimed, Fred Hutch, Amphivena | CD3 × CD33 | Phase I        | Target cell depletion | Cytotoxic effector engagement | Fv + Fv, 2 + 2 | NCT03144245, NCT03516591 |
| BI-836909, AMG-420, RG-6026, RO-7082859 | Amgen, Boehringer Ingelheim | CD3 × CD33 | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv with Fc, 2 + 2 | NCT03647800 |
| EM-901, CC-93269 | Celgene | CD3 × BCMA | Phase I        | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NCT02514239, NCT03836053 |
| ERY-974       | Chugai       | CD3 × GPC3 | Phase I        | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 2 | Company development pipeline |
| GBR-1302      | Glenmark, Harbour BioMed | CD3 × HER2 | Phase I        | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NCT02829372, NCT03983395 |
| GBR-1342      | Glenmark     | CD3 × CD38 | Phase I        | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NCT03091111 |

Continued
Table 4. Continued

| Antibody name     | Organization                  | Targets          | Highest phase | Biological function                          | Type of mechanism     | Format                          | Clinical studies |
|-------------------|-------------------------------|------------------|---------------|----------------------------------------------|-----------------------|---------------------------------|-----------------|
| GEM-333           | GEMoA B, Celgene              | CD3 × CD33       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | scFv + scFv, 1 + 1              | NCT03516760     |
| GEM-3PSCA, GEM3PSCA |                               | CD3 × PSCA       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | scFv + scFv, 1 + 1              | NCT03927573     |
| IGM-2323          | IGM Biosciences               | CD3 × CD20       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 10     | NCT04082936     |
| JNJ-67571244,     | Janssen Research &            | CD3 × CD33       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1        | NCT03915379     |
| JNJ-1244          | Development                   |                  |               |                                              |                       |                                 |                 |
| JNJ-63709178,     | Janssen Research &            | CD3 × CD123      | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1        | NCT02715011     |
| JNJ-9178          | Development                   |                  |               |                                              |                       |                                 |                 |
| JNJ-64007957,     | Janssen Research &            | CD3 × BCMA       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1        | NCT03145181     |
| JNJ-7957          | Development                   |                  |               |                                              |                       |                                 |                 |
| JNJ-63898081,     | Janssen Research &            | CD3 × PSMA       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1        | NCT03926013     |
| JNJ-8081          | Development                   |                  |               |                                              |                       |                                 |                 |
| Orolotamab,       | MacroGenics                   | CD3 × B7-H3      | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fv + Fv with Fc, 1 + 1          | NCT02628535,    |
| MGD-009           |                               |                  |               |                                              |                       | NCT03406949                    |                 |
| Pasotuxizumab,    | Amgen, Bayer                  | CD3 × PSMA       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | scFv + scFv, 1 + 1              | NCT01723475,    |
| AMG-212,          |                               |                  |               |                                              |                       | NCT01723475                    |                 |
| PF-06671008       | Pfizer                        | CD3 × CDH3       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fv + Fv with Fc, 1 + 1          | NCT02659631     |
| PF-06863135,      | Pfizer                        | CD3 × BCMA       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1        | NCT03269136     |
| PF-3135           |                               |                  |               |                                              |                       |                                 |                 |
| REGN-5458         | Regeneron, Sanofi             | CD3 × BCMA       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1        | NCT03761108     |
| RG-6194,          | BTRC-4017A                    | CD3 × HER2       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Not disclosed                  | NCT03448042     |
| TNB-383B          | TeneoBio, AbbVie              | CD3 × BCMA       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + SDA with Fc, 1 + 2        | NCT03933735     |
| XmAb-13676,       | Xencor                        | CD3 × CD20       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1       | NCT02924402     |
| THG-338           |                               |                  |               |                                              |                       |                                 |                 |
| XmAb-14045,       | Xencor, Novartis              | CD3 × CD123      | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1       | NCT02730312     |
| SQZ-622           |                               |                  |               |                                              |                       |                                 |                 |
| XmAb-18087,       | Xencor                        | CD3 × SSTR2      | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1       | NCT03411915     |
| XENP-18087        |                               |                  |               |                                              |                       |                                 |                 |
| HPN-424           | Harpoon                       | CD3 × PSMA × albu-| Phase I       | Target cell depletion                        | Cytotoxic effector engagement | SDA-SDA-scFv, 1 + 1 + 1        | NCT03577028     |
| M-802             | Wuhan YZY Biopharma           | CD3 × HER2       | Phase I       | Target cell depletion                        | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1       | NA              |

Continued
| Antibody name       | Organization                      | Targets                        | Highest phase | Biological function       | Type of mechanism          | Format                          | Clinical studies                  |
|---------------------|-----------------------------------|-------------------------------|---------------|----------------------------|----------------------------|---------------------------------|----------------------------------|
| JNJ-64407564        | Janssen                           | CD3 × GPRC5D                 | Phase I       | Target cell depletion      | Cytotoxic effector engagement (Fab + Fab with Fc, 1 + 1) | NCT04108195, NCT03399799        |
| RG-6160             | Genentech                         | CD3 × FcRH5                  | Phase I       | Target cell depletion      | Cytotoxic effector engagement (Fab + Fab with Fc, 1 + 1) | NCT03275103                     |
| NI-1701, TG-1801    | NovImmune, TG Therapeutics        | CD19 × CD47                  | Phase I       | Target cell depletion      | Enhance phagocytosis (Fc effector) | Fab + Fab with Fc, 1 + 1         | NCT03804996                     |
| MCLA-158            | Merus                             | EGFR × LGR5                  | Phase I       | Target cell depletion      | Cytotoxic effector engagement (Fc effector) | Fab + Fab with Fc, 1 + 1         | NCT03526835                     |
| ZW-49               | Zymeworks                         | HER2 × HER2                  | Phase I       | Target cell depletion      | ADC (Fab + scFv with Fc, 1 + 1) | NCT03821233                     |
| A-319               | Generon (Shanghai)                | CD3 × CD19                   | IND Filed     | Target cell depletion      | Cytotoxic effector engagement (Fab + scFv, 1 + 2) | NCT04056975                     |
| SAR-440234          | Sanofi                            | CD3 × CD123                  | Suspended (1/2) | Target cell depletion      | Cytotoxic effector engagement (Fab + Fv, 1 + 1) | NCT03594955                     |
| AFM-11              | Affimed                           | CD3 × CD19                   | Suspended (1) | Target cell depletion      | Cytotoxic effector engagement (Fv + Fv, 2 + 2) | NCT02106091, NCT02848911        |
| cMet × EGFR ADC     | Sorrento                          | EGFR × cMET                  | Preclinical   | Target cell depletion      | ADC (Not disclosed)         | NA                              |
| APLP2 × HER2 ADC    | Regeneron                         | APLP2 × HER2                 | Preclinical   | Target cell depletion      | ADC (Fab + Fab with Fc, 1 + 1) | NA                              |
| ABP-150             | Abpro                             | Claudin 18.2 × CD3           | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fab + scFv with Fc, 2 + 2) | NA                              |
| ABP-110             | Abpro                             | GPC3 × CD3                   | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fab + scFv with Fc, 2 + 2) | NA                              |
| ABP-140             | Abpro; Luye                       | CEA × CD3                    | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fab + scFv with Fc, 2 + 2) | NA                              |
| ABP-130             | Abpro; Luye                       | CD38 × CD3                   | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fab + scFv with Fc, 2 + 2) | NA                              |
| ABP-100             | Abpro; MSK Cancer Center          | Her2 × CD3                   | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fab + scFv with Fc, 2 + 2) | NA                              |
| CD16 × BCMA × CD200 | Affimed                           | BCMA × CD16 × CD200          | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fv + Fv + Fv, 1 + 2 + 1) | NA                              |
| AFM-26              | Affimed                           | BCMA × CD16                  | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fv + Fv, 2 + 2) | NA                              |
| AFM-24              | Affimed                           | EGFR × CD16                  | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fv + Fv, 2 + 2) | NA                              |
| AFM-21              | Affimed                           | EGFRvIII × CD3               | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fv + Fv, 2 + 2) | NA                              |
| B05/CD3             | Affimed; Immatics                 | MMP1-003/HLA-A*02 × CD3      | Preclinical   | Target cell depletion      | Cytotoxic effector engagement (Fv + Fv, 2 + 2) | NA                              |

Continued
| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|------------------|
| CD3 × FLT3 | Allogene; Maverick; Pfizer | FLT3 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1 | NA |
| Fol-aCD3 | Ambrx | FolRa × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + LIGAND with Fc | NA |
| CD3 × MSLN | Amgen | MSLN × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv with Fc, 1 + 1 | NA |
| CDH19 × CD3 HLE BiTE | Amgen | Cadherin 19 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv with Fc, 1 + 1 | NA |
| CD3 × EGFR Ph-TCB | Amgen; CytomX Therapeutics | EGFR × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 2 + 2 | NA |
| AMX-168 | Amunix | EpCAM × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NA |
| APVO-425 | Aptevo Therapeutics Inc | ROR1 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv with Fc, 2 + 2 | NA |
| ARB-201 | Arbele | Cadherin-17 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv with Fc, 1 + 1 | NA |
| AVA-012 | Avacta | CD22 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Not disclosed | NA |
| CD3 × CD19 | Avacta | CD19 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Not disclosed | NA |
| CD3 × CD123 | Baylor Scott & White Research Institute | CD123 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv with Fc, 2 + 2 | NA |
| CD3 × HER2 | Beijing Hanmi | Her2 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Not disclosed | NA |
| CD3 × DLL3 | Boehringer Ingelheim | DLL3 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv with Fc, 1 + 1 | NA |
| CCW-702 | CIBR*; Scripps | PSMA × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + SMOL* | NA |
| CBA-1535 | Chiome Bioscience | 5T4 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv, 1 + 2 | NA |
| CTX-4419 | Compass Therapeutics | BCMA × NKp30 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 2 + 2 | NA |
| COVA-423I | Covagen; Fred Hutch | CD33 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + SCAFFOLD with Fc, 2 + 2 | NA |
| CD3 × EGFRvIII | Duke University | EGFRvIII × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NA |

Continued
| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|------------------|--------|------------------|
| ESK1          | Eureka; MSK Cancer Center; Novartis | WT1p/HLA-A0201 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NA |
| FPA-151       | Five Prime   | BCMA × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Not disclosed | NA |
| CD3 × CD79b   | Genentech Inc | CD79b × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1 | NA |
| CD3 × HER2 biparatopic GBR-1372 | Genentech | Her2 biparatopic × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + Fab + Fab with Fc, 1 + 1 | NA |
| PM-CD3-GEX    | Glycotope    | TA-MUC1 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 2 + 2 | NA |
| HPN-217       | Harpoon      | BCMA × CD3 × albumin | Preclinical | Target cell depletion | Cytotoxic effector engagement | SDA-SDA-scFv, 1 + 1 + 1 | NA |
| HLX-31        | Henlix; Henlix | Her2 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Not disclosed | NA |
| p95HER2-TCB   | Hospital Vall D’Hebron; MSK Cancer Center; Roche; U. Autonoma de Barcelona | Her2 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 2 | NA |
| E1-3s         | IBC Pharmaceuticals; Immunomedics | Trop2 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + Fab, 1 + 2 | NA |
| CD123/CD3 bsAb | IGM Biosciences | CD123 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + seFv with Fc, 1 + 10 | NA |
| CD38/CD3 bsAb | IGM Biosciences | CD38 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + seFv with Fc, 1 + 10 | NA |
| IPH-61        | Innate Pharma; Sanofi | TAA × NKp46 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 10 | NA |
| CD123-CODV- TCE GNR-047 | INSERM; Sanofi | CD123 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fv + Fv with Fc, 2 + 2 | NA |
| JNJ-0819      | Janssen      | Heme × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + Fab with Fc, 1 + 1 | NA |

Continued
| Antibody name       | Organization                                      | Targets                       | Highest phase | Biological function                      | Type of mechanism         | Format                  | Clinical studies |
|---------------------|---------------------------------------------------|-------------------------------|---------------|----------------------------------------|--------------------------|-------------------------|-------------------|
| Vγ9/Vδ2 TCR × EGFR  | Lava                                             | EGFR × g9/d2 TCR              | Preclinical    | Target cell depletion                  | Cytotoxic effector       | SDA + SDA, 1 + 1       | NA                |
| CD3 × 5T4           | MacroGenics                                       | 5T4 × CD3                     | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fv + Fv with Fc, 1 + 1 | NA                |
| Next-generation CD19 × CD3 DART | MacroGenics                              | CD19 × CD3                     | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fv + Fv with Fc, 1 + 1 | NA                |
| CD123 × CD3 DART    | MacroGenics                                       | CD123 × CD3                   | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fv + Fv with Fc, 1 + 1 | NA                |
| EphA2xCD3 DART      | MacroGenics                                       | Epha2 × CD3                   | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fv + Fv with Fc, 1 + 1 | NA                |
| CD3 × IL13Ra2       | MacroGenics                                       | IL-13Ra2 × CD3                | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fv + Fv with Fc, 1 + 1 | NA                |
| CD3 × ROR1          | MacroGenics                                       | ROR1 × CD3                    | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fv + Fv with Fc, 1 + 1 | NA                |
| CD3 × CD133         | McMaster University; University of Toronto        | CD133 × CD3                   | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fab + scFv, 1 + 1      | NA                |
| h8F4-BiTE           | MD Anderson Cancer Center; Zymeworks             | PR1/HLA-A2 × CD3              | Preclinical    | Target cell depletion                  | Cytotoxic effector       | scFv + scFv, 1 + 1     | NA                |
| ZW-38               | Merck; Zymeworks                                  | CD19 × CD3                    | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fab + scFv with Fc, 1 + 1 | NA            |
| CD3 × HER2          | Molecular Partners                                | Her2 × CD3                    | Preclinical    | Target cell depletion                  | Cytotoxic effector       | SCALFFOLD, 1 + 1       | NA                |
| CD3 × PSMA          | Regeneron                                         | PSMA × CD3                    | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fab + Fab with Fc, 1 + 1 | NA                |
| CD3 × CD20          | Rinat-Pfizer                                      | CD20 × CD3                    | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fab + Fab with Fc, 1 + 1 | NA                |
| CD3 × ROR1          | Scripps Research Institute; Shanghai Yanyi       | ROR1 × CD3                    | Preclinical    | Target cell depletion                  | Cytotoxic effector       | scFv + scFv with Fc, 1 + 1 | NA            |
| B-193               | Shandong Danhong; Shanghai Yanyi                  | CD19 × CD3                    | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Not disclosed          | NA                |
| CD3 × Sialyl-Tn     | Siamab Therapeutics                              | Sialyl-Tn × CD3               | Preclinical    | Target cell depletion                  | Cytotoxic effector       | Fab + scFv with Fc, 2 + 2 | NA                |
| 19-3-19             | SpectraMab                                        | CD19 × CD3                    | Preclinical    | Target cell depletion                  | Cytotoxic effector       | scFv + scFv, 1 + 2     | NA                |
| SV-202              | SYSVAX                                            | CD19 × CD3                    | Preclinical    | Target cell depletion                  | Cytotoxic effector       | SDA + SDA, 1 + 1       | NA                |

Continued
| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|------------------|--------|-----------------|
| SV-201 | SYSVAX | Her2 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | SDA + SDA, 1 + 1 | NA |
| TNB-585 | TeneoBio | PSMA × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + SDA with Fc, 1 + 1 or 1 + 1 + 1 | NA |
| TNB-486 | TeneoBio | CD19 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + SDA with Fc, 1 + 1 | NA |
| TNB-381 M | TeneoBio | BCMA × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + SDA with Fc, 1 + 1 | NA |
| CD3 × CD19 | Tianjin Chase Sun Jinhoda | CD19 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Not disclosed | NA |
| CD3 × MOSPD2 | VBL Therapeutics | MOSPD2 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | scFv + scFv, 1 + 1 | NA |
| M-701 | Wuhan YZY | EpCAM × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NA |
| Y-150 | Wuhan YZY | CD38 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NA |
| CD3 × EMP2 | Wuhan YZY | EMP2 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NA |
| CD3 × EGFR | Wuhan YZY | EGFR × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NA |
| CD3 × CD19 | Wuhan YZY | CD19 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NA |
| CD3 × CD20 | Wuhan YZY | CD20 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NA |
| MS-133 | Wuhan YZY | CD133 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NA |
| XmAb-14484 | Xencor | PSMA × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 1 + 1 | NA |
| YBL-013 | Y-Biologics | PD-L1 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + Fv, 1 + 2 | NA |
| huCD33-BsAb | Y-mAbs | CD33 × CD3 | Preclinical | Target cell depletion | Cytotoxic effector engagement | Fab + scFv with Fc, 2 + 2 | NA |
| BI-905711 | Boehringer Ingelheim | Cadherin-17 × TRAIL-R2 | Preclinical | Target cell depletion | Enhance apoptosis | Fab + scFv with Fc, 2 + 2 | NA |
| Novotarg | Promethera | CD20 × CD95 | Preclinical | Target cell depletion | Enhance apoptosis | Fab + scFv, 1 + 1 | NA |
| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|-----------------|
| ABP-160       | Abpro        | PD-L1 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Not disclosed | NA              |
| BH-29xx       | Beijing Hanmi | PD-L1 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Fab + Fab with Fc, 1 + 1 | NA              |
| IMM-0306      | Gateway Biologics; ImmuneOnco | CD20 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Fab + LIGAND with Fc, 2 + 2 | NA              |
| HMBD-004A     | Hummingbird  | CD33 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Fab + scFv, 1 + 1 | NA              |
| HMBD-004B     | Hummingbird  | BCMA × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Fab + Fab with Fc, 1 + 1 | NA              |
| IMM-2505      | ImmuneOnco   | PD-L1 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Fab + LIGAND with Fc, 2 + 2 | NA              |
| IMM-26011     | ImmuneOnco   | FLT-3 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Fab + LIGAND with Fc, 2 + 2 | NA              |
| IMM-0207      | ImmuneOnco   | VEGF × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | RECEPTOR + LIGAND with Fc, 2 + 2 | NA              |
| IMM-2902      | ImmuneOnco   | Her2 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Fab + LIGAND with Fc, 2 + 2 | NA              |
| IBI-322       | Innovent     | PD-L1 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Not disclosed | NA              |
| NI-1801       | Novimmune    | MSLN × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Fab + Fab with Fc, 1 + 1 | NA              |
| PT-886        | Phanes Therapeutics | Claudin 18.2 × CD47 | Preclinical | Target cell depletion | Enhance phagocytosis | Not disclosed | NA              |
| PT-217        | Phanes Therapeutics | DLL3 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Not disclosed | NA              |
| PMC-122       | PharmAbcine  | PD-L1 × CD47 | Preclinical   | Target cell depletion | Enhance phagocytosis | Not disclosed | NA              |
| DuoHexaBody-CD37 | Genmab       | CD37 biparatopic | Preclinical | Target cell depletion | Fc effector | Fab + Fab with Fc, 1 + 1 | NA              |
| PM-PDL-GEX    | Glycotope    | TA-MUC1 × PD-L1 | Preclinical | Target cell depletion | Fc effector | Fab + scFv with Fc, 2 + 2 | NA              |
| CD38 × IGF-1R | 'rom Group   | CD38 × IGF-1R | Preclinical   | Target cell depletion | Fc effector | scFv + scFv with Fc, 1 + 1 | NA              |

* CIBR, California Institute for Biomedical Research; SMOL, small molecule.
### Table 5. Programs in clinical and preclinical stages for inflammatory conditions

| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|-----------------|
| Ozoralizumab, TS-152, PF-5230896, ATN-103 | Sanofi, Taisho, Eddingpharm | TNF × albumin | Phase III | Half-life extension | Half-life extension | SDA + SDA, 1 + 2 | NCT00959036, NCT01007175, NCT01063803, NCT04077567, NCT02518620, NCT02437890, NCT02309359, NCT02287922 |
| Vobarilizumab | AbbVie; Ablynx | IL-6R × albumin | Phase II | Half-life extension | Half-life extension | SDA + SDA, 1 + 1 | NCT02518620, NCT02437890, NCT02309359, NCT02287922 |
| Romilkimab, SAR-156597 | Sanofi | IL-4 × IL-13 | Phase II | Combinatorial effect | Combinatorial effect | Fab + Fv with Fc, 2 + 2 | NCT01529853, NCT02345070, NCT02921971 |
| M-1095, ALX-0761, MGD-010, PRV-3279 | Avillion; Merck Serono | IL-17A × albumin × IL-17F | Phase II | Combinatorial effect | Combinatorial effect | SDA + SDA, 1 + 1 + 1 | NCT03384745, NCT02156466, NCT02376036 |
| AMG-570, MEDI-0700 | Amgen, Viela Bio, AstraZeneca | BAFF × ICOSL | Phase I | Combinatorial effect | Combinatorial effect | Fab + PEPTIDE with Fc, 2 + 2 | NCT02618967, NCT03156023, NCT04058028, Company development pipeline |
| Tibulizumab | Eli Lilly | BAFF × IL-17A | Phase I | Combinatorial effect | Combinatorial effect | Fab + scFv with Fc, 2 + 2 | Company development pipeline |
| JNJ-61178104 | Janssen Research & Development | TNF × IL-17A | Phase I | Combinatorial effect | Combinatorial effect | Fab + Fab with Fc, 1 + 1 | NCT02758392 |
| ONO-4685 | Ono | CD3 × PD-1 | Phase I | Dominant negative | Dominant negative | Fab + Fab with Fc, 1 + 1 | NCT04079062 |
| ES-210, APVO-210 | Aptevo Therapeutics | CD86 - IL10 | Phase I | Tissue specificity | Tumor or tissue localization Fc effector | Fab + Fab with Fc, 1 + 1 scFv + scFv with Fc, 2 + 2 | NCT03768219 |
| AM-201 | AbClon | IL-6R × TNF | Preclinical | Anti-inflammation | Combinatorial effect | Fab + SCAFFOLD with Fc, 2 + 2 | NA |
| IL4Ralpha/IL-5 bsAb | arGEN-X | IL-4Ra × IL-5 | Preclinical | Anti-inflammation | Combinatorial effect | Fab + scFv with Fc, 2 + 2 | NA |

Continued
Table 5. Continued

| Antibody name | Organization | Targets | Highest phase | Biological Function | Type of mechanism | Format | Clinical studies |
|---------------|--------------|---------|---------------|---------------------|------------------|--------|-----------------|
| BH-1657       | Beijing Hanmi | TNF $\times$ IL-17A | Preclinical | Anti-inflammation | Combinatorial effect | Fab + Fc with Fc, 1+1 | NA |
| IL-4 $\times$ IL-13 | Beijing VDJBio | TNF $\times$ IL-17A | Preclinical | Anti-inflammation | Combinatorial effect | Fv + Fc with Fc, 1+1 | NA |
| IL-1 $\times$ TNF $\alpha$ | Beijing VDJBio | TNF $\times$ IL-17A | Preclinical | Anti-inflammation | Combinatorial effect | Fab + Fc with Fc, 2+2 | NA |
| CMX-02        | Complix      | TNF $\times$ IL-23 | Preclinical | Anti-inflammation | Combinatorial effect | Fab + scFv with Fc, 2+2 | NA |
| IL-17A $\times$ albumin | Intarcia | TNF $\times$ IL-17A | Preclinical | Tumor or tissue localization | Combinatorial effect | Fab + scFv with Fc, 2+2 | NA |
| PT-001        | Pandion      | MAdCAM $\times$ PD-1 | Preclinical | Half-life extension | Combinatorial effect | MAdCAM $\times$ PD-1 | NA |
| ALXN-1720     | Alexion      | C5 $\times$ albumin | Preclinical | Half-life extension | Combinatorial effect | C5 $\times$ albumin | NA |
| YBL-004       | Y-Biologics  | MArcAM $\times$ PD-1 | Preclinical | Half-life extension | Combinatorial effect | MArcAM $\times$ PD-1 | NA |

**SDA-based.** Two different VHHs can be fused to form a bsAb [4]. This format may be the smallest bsAb format with molecular weight approximately 25 KD. It has been reported that two different VHHs can be fused to coiled-coil peptide to form Combody. The peptide facilitates the oligomerization of the antibody and renders the antibody avidity effect [5]. Two different VHH can also be engineered on the N-terminus of CH1 and CL to form a Fab-like 1+1 bsAb fragment [6].

**ScFv-based.** Bispecific T cell engager (BiTE), one of the formats used to redirect T cells to tumor cells, comprises two tandem linked scFv: one scFv against a tumor-associated antigen and another binding to CD3 on T cells. The structure and mechanism of BiTE was well reviewed by Wolf [7]. Two scFvs can also be indirectly linked, such as via a CL, to form a bsAb in scFv-CL-scFv format [8]. Due to aggregative tendency of scFv, various techniques were employed to stabilize scFv. Brolucizumab (Beovu) was engineered using scFv-λ cap platform [9]. Similar technology was also used to build multispecific antibody (msAb)-based therapeutics by cognate heterodimerization (MATCH) [10], where up to four distinct binding sites can be integrated into a multispecific Fv- or scFv-based molecule.

**Fv-based.** A diabody molecule is formed by two polypeptides: one polypeptide contains VH$a$ and VL$b$; another polypeptide contains VH$b$ and VL$a$. Due to the short linker, VH$a$ associates with VL$b$ and similarly VL$b$ associates with VH$b$ to form 1+1 bsAb fragment. A diabody-based bispecific format is called dual-affinity retargeting antibody or DART [11–13]. DART molecules may contain Fc domain to extend in vivo half-life and grand effect functions. TandAb is another Fv-based bispecific fragment: two polypeptides are forced to fold in a head-to-tail fashion to form 2+2 bsAb fragment [14].

**Combination.** In a native antibody, VH and VL are on the N-terminus of Fab region and CH1 and CL on C-terminus. It was found that CH1 and CL can also facilitate the association of VH and VL on C-terminus of a Fab-Fv fusion protein. This Fab directed VH-VL association can be further improved by introducing a disulfide bond between the VH and VL on C-terminus of a Fab-Fv format. Two VHHs can be fused to a bsAb in SDA-based format [15]. A VH on the C-terminus of a Fab-Fv may associate with a C-terminal VL on another Fab-Fv to form 2+2 tetramer Fab-Fv [16]. Similarly, a scFv can be fused on the C-terminus of a Fab to form Fab-scFv fusion proteins. The so-called bbody has one Fab with one scFv, and “tribody” has one Fab with two scFvs [17]. A trobody can be either bispecific or trispecific, depending on the specificity of the two attached scFvs. A VHH can be fused to a light chain C-terminus of a Fab to form 1+1 bsAb fragment [18]. It was reported that three tandem linked VHHs can be fused with a scFv to form 1+3 bsAb fragment [19]. A bsAb fragment containing a VHH or scFv specific to human serum albumin is a common strategy to extend serum half-life of such molecules.
Table 6. Programs in clinical and preclinical stages for other conditions

| Antibody name        | Organization                                | Targets                        | Highest phase | Biological function                          | Type of mechanism                     | Format                        | Conditions                          | Clinical studies                |
|----------------------|---------------------------------------------|--------------------------------|---------------|-----------------------------------------------|---------------------------------------|-------------------------------|-----------------------------------|-------------------------------|
| Faricimab, RG-7716,  | Roche, Chugai Pharmaceutical                | VEGF × ANGPT2                  | Phase III     | Anti-angiogenesis                             | Combinatorial effect                  | Fab + Fab with Fc, 1 + 1       | Ocular, diabetic retinopathy     | NCT01941082, NCT02484690,    |
| RO-6867461           |                                             |                                |               |                                               |                                       |                               |                                   | NCT02699450, NCT03038880,      |
|                      |                                             |                                |               |                                               |                                       |                               |                                   | NCT03622580,                  |
|                      |                                             |                                |               |                                               |                                       |                               |                                   | NCT03622593,                  |
|                      |                                             |                                |               |                                               |                                       |                               |                                   | NCT03823287,                  |
|                      |                                             |                                |               |                                               |                                       |                               |                                   | NCT03823300,                  |
|                      |                                             |                                |               |                                               |                                       |                               |                                   | NCT03814291                   |
| IBI-302              | Innoven                                     | VEGF × complement              | Phase I       | Anti-angiogenesis; anti-inflammation          | Combinatorial effect                  | Not disclosed                  | Ocular                            | NCT02255760,                  |
|                      |                                             |                                |               |                                               |                                       |                               |                                   | NCT02696902                   |
|                      | MedImmune                                    | PcrV × PsI                     | Phase II      | Combinatorial effect                          | Combinatorial effect                  | Fab + scFv with Fc, 2 + 2       | Antibacterial                   | NCT02508155,                  |
|                      |                                             |                                |               |                                               |                                       |                               |                                   | NCT03755934                   |
|                      | AstraZeneca                                  | NGF × TNF                      | Phase II      | Combinatorial effect                          | Combinatorial effect                  | scFv + RECEPTOR with Fc, 2 + 2  | Analgesic drugs                 |                               |
|                      |                                             |                                |               |                                               |                                       |                               |                                   |                               |
|                      | 10E8.4/iMab                                   | HIV-1 Env × CD4                 | Phase I       | Broaden protection                            | Combinatorial effect                  | Fab + Fab with Fc, 1 + 1        | HIV-1                            | NCT03875209                   |
|                      | TaiMed, Aaron Diamond AIDS Research Center   |                                |               |                                               |                                       |                               |                                   |                               |
|                      | Sanofi, NIH                                  |                                |               |                                               |                                       |                               |                                   |                               |
|                      | MGD-441236                                   | HIV-1 Env triparatopic protein  | Phase I       | Combinatorial effect                          | Combinatorial effect                  | Fab + Fv with Fc, 1 + 1 + 1     | HIV-1                            | NCT03705169                   |
|                      | MacroGenics, NIAID                           | CD3 × HIV-1 Env protein         | Phase I       | Target cell depletion                         | Cytotoxic effector engagement         | Fv + Fv with Fc, 1 + 1 + 1      | HIV-1                            | NCT03570918                   |
|                      | Genentech                                    |                                |               |                                               |                                       |                               |                                   |                               |
|                      | BFKB-8488A, RG-7992                          | FGFR1 × beta-Klotho             | Phase I       | Tissue specificity                            | Tumor or tissue localization          | scFv + scFv with Fc, 1 + 1     | Diabetes                         | NCT02593331,                  |
|                      |                                               |                                |               |                                               |                                       |                               |                                   | NCT03060538                   |
|                      | ABP-201                                      | VEGF × ANGPT2                  | Preclinical   | Anti-angiogenesis                             | Combinatorial effect                  | scFv with Fc, 2 + 2             | Ocular                           |                               |
|                      | AbMed                                        |                                |               |                                               |                                       |                               |                                   |                               |
|                      | SL-634                                       | VEGF × ANGPT2                  | Preclinical   | Anti-angiogenesis                             | Combinatorial effect                  | Not disclosed                  | Ocular                           |                               |
|                      | University of Colorado System, Kodaki Sciences |                                |               |                                               |                                       |                               |                                   |                               |
|                      | KSI-501                                      | VEGF × IL-6                    | Preclinical   | Anti-angiogenesis, anti-inflammation          | Combinatorial effect                  | Fab + RECEPTOR with Fc, 2 + 2  | DME, Uveitis                     | NA                            |
|                      | Humabs BioMed                                | Zika E protein                 | Preclinical   | Broaden protection                            | Biparotopic                           | Fab + scFv with Fc, 1 + 1      | Infection                       | NA                            |
|                      |                                              | biperatopic                    |               |                                               |                                       |                               |                                   |                               |
|                      | FIT-1                                        | VEGF × ANGPT2                  | Preclinical   | Anti-angiogenesis                             | Combinatorial effect                  | Fab + scFv with Fc, 1 + 1      | Infection                       | NA                            |
|                      | Aaron Diamond AIDS Research Center; TaiMed   | HIV gp120 × CD4                | Preclinical   | Broaden protection                            | Combinatorial effect                  | Fab + Fab with Fc, 2 + 2       | Infection                       | NA                            |
|                      | Visterra, Vir Biotechnology                  | RSV F × RSV G                  | Preclinical   | Broaden protection                            | Combinatorial effect                  | Fab + scFv with Fc, 1 + 1      | Infection                       | NA                            |

Continued
### Table 6. Continued

| Antibody name | Organization | Targets | Highest phase | Biological function | Type of mechanism | Format | Conditions | Format | Conditions | Format | Conditions | Format | Conditions |
|---------------|--------------|---------|---------------|---------------------|-------------------|--------|------------|--------|------------|--------|------------|--------|------------|
| Dual FZD and LRP5/6 agonist | ABL Therapeutics | ABL Bro | Preclinical | Neutralizing | Trojan horse | scFv | Fab with Fc, 1 | 1 + 1 + 1 |
| ATV/BACE1/Tau Domali | Kymab Ltd | KY-1049 | Preclinical | Neutralizing | Trojan horse | Fab with Fc, 1 | 1 + 1 + 1 |
| Bispecific fully human IgG | Shire plc | | | | | | |

**Fc-containing bispecific antibodies**

The Fc region, part of natural antibody, is homodimer of two polypeptide chains. Depending on the isotype of the antibody, each comprising two to three heavy chain constant domains (CH2, CH3, and CH4). The Fc region not only imparts an antibody effector functions due to FcγR binding and complement-binding but also extends its in vivo half-life via FcRn binding.

When two different heavy chains and two different light chains of IgG antibodies are expressed in one cell, these different heavy chains and light chains may scramble randomly, possibly to form 10 different IgG antibody molecules. Among these, statistically only 12.5% would be desired bsAb. For symmetric Fc-containing bispecific formats, a challenge is to avoid heavy chain/light chain mispairing. For asymmetric Fc-containing bispecific formats, an additional challenge is to force heterodimeric heavy chain formation.

Forming heterodimeric Fc can be achieved by engineering the interface of two CH3 (for IgG) or CH4 (for IgM or IgE) domains, by changing size (knob-into-hole) [20–22] and electrostatic steering [23]. Computational methods have been used to identify the mutations that facilitate heterodimeric association [24, 25]. Several groups also used the interface of different Ig proteins to design the heterodimeric Fc. Davis et al. developed derivatives of human IgG and IgA CH3 domains composed of alternating segments of human IgA and IgG CH3 sequences [26]. Skegro et al. grafted some residues from T cell receptor (TCR) constant region to CH3 of IgG1 or CH4 of IgM [27]. An alternative strategy is to purify heterodimer from unwanted homodimers. With the modification on CH3 domain, the heterodimer and homodimer have different affinity binding on Protein A, and the bsAb with heterodimeric Fc can be isolated [28].

In order to ensure cognate heavy chain and light chain pairing, several strategies have been reported. The first strategy is to use SDA, scFv, or scFab as antigen-binding building blocks. In addition, single-chain IgG has been reported [29], where two heavy chains, two light chains, and three linker sequences were expressed from one gene. Protease cleavage sites were integrated into these linkers, allowing protease digestion to cleave the linkers. The second strategy is using common light chain or common heavy chain. In the case of common light chain, an identical light chain is used as the partner for two different heavy chains [30–33]. Common heavy chain was also reported in a κλ-body case [34]. The third strategy is to modify the interface of VH-CH1 and VL-CL, including developing orthogonal Fab interface [35, 36], altering the location of interchain disulfide bond [37–39], and addition of charged pairs and knob-into-hole [40, 41]. These strategies usually involve changes on both VH-CL interface and CH1/CL interface. Yet, Bonish et al. reported that the preferential association can be achieved by only engineering the CH1/CL interface [42]. To associate cognate VH and VL, the CH2 domain from IgM or IgE have been engineered to form heterodimer to replace CH1/CL [43] [Dong, WO2017011342].

There are additional strategies to avoid heavy chain/light chain mispairing. Schaefer et al. described a CrossMab
Figure 2. The classification of bsAb formats based on assembly of antibody fragments as building blocks. The first row and column list the five basic building blocks (SDA, Fv, scFv, Fab, scFab). The different color and shape of VH and VL represent their origins from different parental antibodies. The assembly of different building blocks creates various bsAb formats classified into 30 groups. An exemplary format and its molecular weight of each group are listed. The diagonal line divides the formats into bispecific formats without Fc (top right with number 1-15) and bispecific formats with Fc (bottom left with number 16-30): 1, tandemly linked SDAs; 2, a SDA tandemly linked on the VH of a Fv; 3, a SDA tandemly linked on the VH of a scFv; 4, two SDAs are separately linked on the carboxyl-terminus of constant domain of a Fab; 5, a SDA tandemly linked on the VL of a scFab; 6, the VHs and VLs of two Fvs cross pair to each other to form diabody; 7, a scFv tandemly linked on the VH of a Fv; 8, the VH and VL of a Fv each linked on the carboxyl-terminus of CH1 and CL of a Fab; 9, the VH of a Fv linked to the CH1 of a scFab; 10, two tandemly linked scFvs; 11, a scFv linked on the VH of a Fab; 12, a scFab.
As a small, flexible, and modular antigen-binding site, it is obvious that SDA or VHH can be fused to N-terminus or C-terminus of heavy chain or light chain of an IgG antibody. As shown in Fig. 3, SDA can also be inserted in other fusion sites. Shen et al. reported that a SDA antibody can be fused with VL to form 2+2 bispecific format [46, 47]. Shi et al. used two different SDAs to replace VH and VL of an IgG, respectively, to form 2+2 bivalent or multivalent antibody [48]. In addition to variable domain, CH3 domain on Fc has been engineered as binding site [49]. Broadly speaking, the engineered CH3 is a SDA that can be integrated into a bispecific format, such as IgG-like BsAb [50].

**Fv-based.** VH and VL domains are tandemly linked with VH and VL of another IgG antibody, to form a 2+2 bispecific IgG format, named as “dual variable domain immunoglobulin” or DVD-Ig [51]. Fab + Fv-based 1+2 format called mAb-Fv [24] was mentioned above. Fv can also replace CH2 domain to form a 1+2 bispecific format called TriFab [52]. Seifert et al. employed diabody format combined with heterodimeric CH2 from IgM or IgE to construct 2+2 bispecific format [43]. Aforementioned WuXiBody® formats are also Fv-based formats, including 1+1, 1+2, and 2+2 formats.

**ScFv-based.** There are many Fc-containing bispecific formats comprising of scFv, although scFv is prone to aggregate. A scFv can be fused with heavy chain of IgG to form symmetric 2+2 bsAb [53], called Morrison format. Morrison format is one of the earliest bispecific formats that have still been widely used. ScFv can also be fused with light chain [54]. To form 1+1 format, several groups used scFv to replace one of the Fabs on an IgG and used engineered Fc heterodimer [55, 56]. Two different scFvs can be used to replace both Fabs on an IgG antibody to form 1+1 bispecific format [57, 58]. ScFv can also be placed in the hinge region or CH3 domain to form 2+2 formats [59, 60]. Kim et al. fused scFv with CH1-CH2-CH3 and co-expressed LC domain, potentially masking the hydrophobic part of scFv to make the molecule more stable [61].

**Fab/scFab-based.** Several Fab- or scFab-based bispecific formats have been reported. Fab-based CrossMab, a 1+1 bispecific IgG format [44], was mentioned above. Fabs-intandem immunoglobulin (FIT-Ig) is a format where a Fab is fused to the N-terminus of another IgG antibody: the light chain of the Fab is fused with the heavy chain of the IgG, and FD chain of the Fab and light chain of the IgG are separate polyopeptides. These three different polyopeptides can be co-expressed from single cells and be assembled to form the bsAb [62]. In theory, the FD of antibody A and the light chain of antibody B can associate to each other to form a hybrid Fab, but this hybrid Fab can be removed in Protein A purification step. Bostrom et al. described a two-in-one bsAb, a bsAb in regular IgG form, and each arm can bind two distinct antigens [63–65]. Hu et al. even developed a four-in-one antibody [66]. Strop et al. showed that making mutations in hinge region and CH3 domain of human IgG1 and IgG2 could facilitate heterodimerization of heavy chain. Two parental antibodies can be expressed and purified separately and mixed together under appropriate redox conditions, resulting in formation of a stable bsAb [67]. Labrijn et al. reported a similar platform, later called DuoBody [68]. scFab can be used to construct 1+1 format [69], and it can also be used as one of the building blocks to construct tetraspecific antibody [70].

**Other binding modalities**

As mentioned earlier, peptides, ligands, receptors, and different protein scaffolds, either native form or engineered form, can be used as antigen-binding sites. The non-antibody scaffolds include Adnectin, DARPin, Affilins, alpha helix scaffold, avimer, Centyrin, Duocalin, Ecallantine, Fynomer, microprotein, peptide, Protein A domain, trimeric, TCR, etc. There are numerous possibilities to tandemly linked with a scFv; 13, two tandemly linked Fabs: the light chain of one Fab linked with the heavy chain of another Fab and vice versa; 14, a scFv linked on the VH of a Fab; 15, tandemly linked two scFab; 16, two tandemly linked SDA on Fc to form homodimer; 17, a SDA and the VH of a Fv linked to the FcA to pair with the VL of a Fv linked to another FcB to form heterodimer; 18, a diabody on FcA to pair with FcB to form heterodimer; 19, a SDA on FcA to pair with a scFv on FcB to form heterodimer; 20, a scFv and the VH of a Fv linked to the FcA to pair with the VL of the Fv linked with FcB to form heterodimer; 21, two scFv tandemly linked to the amino- and carboxyl-terminal of a Fc to form homodimer; 22, a SDA tandemly link to the light chain of a IgG to form homodimer; 23, a TCR constant domain anchored Fv linked to FcA to pair with a half IgG with FcB to form heterodimer (WuXiBody®); 24, a scFv linked FcA to pair with a half IgG with FcB to form heterodimer; 25, two tandemly linked Fabs (the light chain of a Fab linked on the heavy chain of another Fab) on Fc to form homodimer (FIT-Ig); 26, a SDA on FcA to pair with a scFv on FcB to form heterodimer; 27, a scFv and the VH of a Fv linked to FcA to pair with the VL linked on FcB to form heterodimer; 28, a scFv linked on FcA to pair with a scFv linked on FcB to form heterodimer; 29, a half IgG with FcA paired with scFv linked on FcB to form heterodimer; 30, two scFab each linked on FcA and FcB to form heterodimer. Above mentioned FeA and FeB are engineered Fc pair to facilitate Fc heterodimerization.
generate bispecific formats using these binding modalities. Recent examples include peptide [71], VEGF receptor [72], TCR [73], and single-chain TRAIL [74].

In the new paradigm of bsAbs, many novel formats have been designed and tested. The general goal is to design a molecule to enable novel therapeutic mechanisms and to make homogeneous product in large scale to meet the need of clinical development and commercial manufacturing. Additionally, more multispecific formats have been reported in the recent years, including trispecific [75], tetraspecific [70], and pentaspecific [71].

THE RESURGENCE OF BISPECIFIC ANTIBODIES

During the last few years, the number of clinical studies using bsAbs has increased exponentially (Fig. 4A). In fact, this increase in 2014 matched with the launch of Blincyto (Amgen), the first commercialized BiTE for the treatment of acute lymphoblastic leukemia. However, it was not until 2017 that another bsAb, Hemlibra (Roche), was launched for the treatment of hemophilia A. Currently, most bsAbs in clinical development are in early studies (67% in Phase I, 25% in Phase II) with only five products in Phase III studies (Fig. 4B). The majority of clinical stage bsAbs (~84%) are designed to treat cancer, especially solid tumors, breast cancer, and acute myeloid leukemia. Nevertheless, there are some products designed to treat other conditions such as rheumatoid arthritis or autoimmune diseases (Fig. 4C). The company with more bsAbs under active development is Amgen, followed by MacroGenics and then Lilly, Janssen, Roche, Sanofi, and Xencor. The strategy in nearly half of the developing bsAbs is to deplete the malignant cells by engaging cytotoxic effector cells including T or natural killer (NK) cells using CD3 or FcGR3A (CD16) targeting arms. Another commonly used strategy is to identify tumor or tissue-specific markers to act only in the affected areas. For that, many companies have designed their own technology to manufacture bsAbs, including Amgen’s BiTE, MacroGenics’ DART, or Roche’s CrossMab platforms.

Accordingly, with the increase in bsAb development, the number of deals (excluding mergers and acquisitions) have focused on clinical stage bsAbs resulting in an increase of 140% in the last 3 years, while the total disclosed deal value decreased by 50%, from $3.2 billion to $1.6 billion; $6.8 billion is recorded across the whole period (Fig. 5A). From these deals, nine were worth more than $100 million, and most were structured with milestones, signifying the balancing of risk and reward between the partners. Sanofi and Regeneron’s 2015 co-development agreement focused on various antibodies, including CD3 × MUC16 (REGN-401) and CD3 × BCMA (REGN-5458) (Fig. 5B). From these deals, $3.8 billion were spent on oncology followed by $1 billion on infection diseases from a total of $6.8 billion, probably due to the clinical and commercial potential of treating patients in these disease areas with bsAbs (Fig. 5C).

The global market of bsAb was worth $0.46 billion in 2018, which was dominated almost equally by Blincyto ($230 million) and Hemlibra ($229 million). As predicted, the market for Hemlibra will boom in the next few years and grow up to $3.96 billion by 2024. Instead, Blincyto will only have a moderate increase. With the massive sales growth of Hemlibra and the potential approval of new entrants, for instance, faricimab, gremubamab, MCLA-117, and XmAb-14045, the bsAb market will surpass $5.43 billion in 2024 (Fig. 5D).

BIOLOGY DRIVES DEVELOPMENT OF VARIOUS BISPECIFIC ANTIBODIES

Most human diseases are complex, often driven by multiple redundant or distinct mechanisms; thereby single-target approach such as mAb may not be sufficient to achieve optimal therapeutic efficacy. Especially, many therapeutic concepts need physical linkage of two or more targets. In this case, bsAbs or msAbs targeting two or more targets may offer novel therapeutic applications that are difficult to achieve by mAbs. In a recent comprehensive review article, Aran Labrijn, Maarten Janmaat, Janice Reichert,
Figure 4. Statistics showing the booming of bsAb programs. A). The number of clinical studies associated with bsAb in the past fourteen years (up to September 2019). The bsAb programs classified based on B) different clinical stages and C) different disease areas. Data source: Cortellis™ Competitive Intelligence (CCI) and Cortellis™ Drug Discovery Intelligence (CDDI, formerly Integrity) as of Sept 23, 2019.

Figure 5. Licenses and market analysis for bsAb programs. A). Licenses for clinical stage bsAbs. Line represents number of license signed each year. Blue and yellow bars represent the largest deal and total deal values for each year, respectively. B). The largest deals signed from 2014 to 2018. C). Deal values in disease area. D). BsAb market size in 2018 and forecast in 2024. Data source: Cortellis™ CCI as of Sept 23, 2019.

and Paul Parren thoroughly reviewed global bispecific antibody clinical pipeline using a mechanistic lens [2]. Based on the analysis using Cortellis™, a Clarivate Analytics solution, by the end of September 2019, there are 176 bsAbs or bifunctional proteins with target disclosed under active preclinical development, compared to 119 in clinical development for cancer, autoimmune, and other indications (including IND filed—Phase III—and two programs on clinical hold). This highlights the increased interest of exploring bsAbs as a venue to develop novel antibody-based therapeutics. The most frequently studied target pairs of those bspecific antibodies and the number of molecules being explored are illustrated in a network graph (Fig. 6). We took a step further and analyzed the disease areas cov-
Figure 6. A network graph characterizing the target pairs of most bispecific programs in both preclinical and clinical investigations. Each node in the network is one target, and each edge connecting two nodes represents one bispecific program. The circular edges are biparatopic programs. The node size shows the degree of a particular target being paired with other different targets. The colors of the edges are marked in black if only one program is available for that particular pair, otherwise in red if more than one are being explored. The popularity of that bispecific program is reflected from the thickness of the red edges. Source data are from Cortellis™ (Table 1-4). Tri-specific and albumin-relevant bispecific programs are not included. The albumin-relevant tri-specific are analyzed as bispecific projects.

Bispecific antibodies for cancer treatment

According to the Cortellis™ analysis, the bsAb pipeline is composed predominantly by programs for cancer treatment, with 99/119 programs in clinical stages and 153/176 preclinical programs (Fig. 4C). As reviewed by Hanahan D. and Weinberg R.A., there are eight hallmarks of cancer, and targeting these biological capabilities of cancer cells may lead to new therapeutic options for cancer [76]. Therefore, based on the biological functions, we categorize the bsAb programs into the following groups: anti-angiogenesis, anti-tumorigenesis, enhancing tumor immunity, modulating tumor microenvironment (TME), and depletion of target cells.

Anti-angiogenesis. As angiogenesis plays an essential role in promoting tumor progression and metastasis, anti-angiogenesis for cancer treatments have been extensively explored. Though several therapies, such as bevacizumab (anti-VEGF) and ramucirumab (anti-VEGFR2), have been approved to treat several types of tumors, only moderate levels of antitumor activity were observed. Along with the booming of bispecific programs and better understanding of the angiogenesis process, new generation of anti-angiogenesis treatments are emerging. As shown in Supplementary Table S1 available online at ABT Online, 12 programs are under active development. Majority of the programs are focusing on improving the anti-angiogenesis effect by combinatory targeting two or even three molecules that are involved in angiogenesis, such as VEGF, VEGFR2, DLL4, and ANGPT2.

Dilpacimab (AbbVie) targeting DLL4 and VEGF is one of the most advanced programs in this category. DLL4-Notch signaling plays a critical role in angiogenic sprouting, and DLL4 blockade alone has shown inhibition...
in tumor growth [77, 78]. Dilpacinib was designed to co-inhibit both DLL4 and VEGF signaling to achieve more prominent antitumor efficacy [79]. Dilpacinib was generated using DVD-Ig platform with the variable domain (VD) of anti-DLL4 located at the outer position and anti-VEGF VD located at the inner position. Interestingly, in the presence of VEGF, dilpacinib showed 20–50× enhanced capability of blocking DLL4 signaling, which may be due to the VEGF homodimerization-mediated cross-linking of dilpacinib, then enhanced its binding avidity to DLL4 on the cell surface, and promoted the downregulation of DLL4 [79]. Considering higher levels of VEGF at tumor sites than in peripheral circulation, this unique characteristic of dilpacinib may conditionally enhance DLL4 neutralization activity only at tumor sites. Currently, the treatment of dilpacinib along or in combination with chemotherapy in patients with advanced solid tumor have shown acceptable safety profile and demonstrated preliminary antitumor efficacy [80, 81].

**Anti-tumorigenesis.** Anti-tumorigenesis by targeting oncogenic receptors is another well-validated anticancer treatment. Trastuzumab targeting Her2 was approved to treat Her2-overexpressing breast cancer in 1999. Later, pertuzumab recognizing a different epitope of Her2 was approved in 2012 for treatment of patients with Her2-positive metastatic breast cancer in combination with trastuzumab and chemotherapy. To develop an ideal combinatorial treatment with trastuzumab and pertuzumab, a handful of biparatopic Her2 bsAb programs are under early clinical testing. ZW25, a biparatopic Her2 bsAb designed based on Azymetric platform, able to bind domains 2 and 4 of extracellular region of HER2 simultaneously to promote internalization of HER2 and to inhibit HER2/HER3 heterodimer formation, demonstrated promising clinical efficacy in a Phase I study (ESMO-Asia 2019). Additionally, several other oncogenic targets are also under evaluation, such as EGFR, Her3, cMET, and lipoprotein receptor-related proteins (LRP) 5/6. Leading players in this area are Merus (Her3 × Her2), Jiangsu Alphamab (Her2 biparatopic), Zymeworks (Her2 biparatopic), Janssen (EGFR × cMET), and EpimAb (EGFR × cMET), followed by Beijing Mabworks, Boehringer Ingelheim (BI), Molecular Partners, etc. (Table 1).

In collaboration with Gemmab, Janssen has developed JNJ-61186372 to concurrently block both EGFR and cMET pathways for treatment of patients who are resistant to EGFR tyrosine kinase inhibitors (TKIs). It has been shown that JNJ-61186372 not only effectively blocks the ligand binding-induced EGFR and cMET activation but also promotes the downregulation of both EGFR and cMET. To further enhance its antitumor potency, the antibody-dependent cellular cytotoxicity (ADCC) function of JNJ-61186372 is augmented by production in a fucosylation defective CHO cell line [82]. In the first-in-human (FIH) Phase I study, JNJ-61186372 has been tested on 142 patients who were progressed after EGFR TKI therapies and has shown promising antitumor activity with ~30% partial response rate.

Though most of the companies are focusing on modulating the signaling of the well-validated ErBB family members, Boehringer Ingelheim is developing a first-in-class biparatopic antibody to block the function of LRP5/LRP6 and Wnt/β-catenin pathway. LRP5/LRP6 forms trimeric complex with the serpentine receptor Frizzled and Wnt and mediates the stabilization of β-catenin, the transcriptional activator of the Wnt targeting genes. Aberrant Wnt/β-catenin pathway activation can contribute to the carcinogenesis and has been observed in many types of tumors. It has been suggested that LRP5/LRP6 can interact with different Wnt ligands at different domains, and mAbs blocking different domains showed different profile [83]. Therefore, BI generated the LRP5/LRP6 biparatopic nanobody, BI 905677, with high affinity and complete blockage of the binding of Wnt ligands to LRP5/LRP6, thereby inhibiting the Wnt-mediated cancer cell proliferation and survival [84]. This molecule is currently at Phase I in patients with different types of solid tumors.

To further expand the antitumor activity, strategies in combining the blockage of both angiogenic and tumorigenic pathways have been exploited, such as targeting VEGF and cMET (Merus), as well as Her3 and LGALS3BP (MediaPharma). Both programs are still at preclinical stage (Table 1).

**Enhance tumor immunity.** Though the idea of immunotherapy dates back to the 1890s, it was not until the 2010s when it had significant breakthrough with ipilimumab launched in 2011 and Keytruda and Opdivo in 2014. The aim of the immunotherapy is to boost the patients’ own immune system to generate antitumor T cell responses. This can be achieved by either blocking the inhibitory signals, such as CTLA-4 and PD-1, or enhancing the co-stimulatory signals, such as 4-1BB and OX40. Till today, anti-CTLA-4 and anti-PD(L)1 treatments have shown promising efficacy and revolutionized cancer treatment. Nevertheless, only 10–30% of the patients benefit from the treatment [85, 86].

The immune system is a fine-tuned system, with redundancy in most of the regulatory pathways to avoid damage to the host while it remains effective to clear infection and tumor cells. To further improve the antitumor efficacy of anti-CTLA-4 and anti-PD(L)1 therapies, several strategies are being evaluated, including combining the anti-angiogenesis treatment with anti-PD-1 treatment (VEGF + PD-1) and combining the blockage of multiple immune checkpoints (CTLA-4 + PD-1 [87], PD-1 + LAG-3, etc.). Though the additive effect can be achieved by combining two mAbs, bsAbs have the advantages in development as a single molecule entity, sometimes may even have synergistic efficacy. As of September 2019, there are 11 bsAbs targeting multiple inhibitory immune modulatory pathways such as PD-1, CTLA-4, LAG-3, TIM-3, etc. under active development at clinical and 13 at preclinical stage (Table 2). For example, the co-expression of PD-1 and LAG-3 on tumor-infiltrating lymphocytes identifies the tumor-specific T cells [88], which are mostly dysfunctional [89]. The co-treatment of anti-PD-1 and anti-LAG-3 can effectively restore the T cell function [90] and have showed antitumor activity in PD-1-resistant patients [91]. Based on this fact, several companies are evaluating PD-1 × LAG-3 bsAbs. Some of the molecules
represent preferential binding on the double-positive T cells and are more effective in upregulating the T effector cell function, as compared to the combination of the two parental antibodies [WO2017019846; WO2018134279; WO2018185043; WO2019158942].

Despite the success achieved by the immune checkpoint inhibitors, the development of co-stimulatory signal agonists was hindered by the intriguing balance between safety and efficacy. For instance, the co-stimulatory molecule 4-1BB is a promising target for cancer immunotherapy, as it can activate T cells, NK cells, and other immune cells and has been clinically validated in CAR-T therapies to sustain T cell activation. However, the clinical development of anti-4-1BB monoclonal antibodies was stagnated by either liver toxicity or lack of efficacy [92]. To minimize the safety issue associated with the systemic activation of 4-1BB, strategies have been employed to localize the activation of 4-1BB at tumor site. Roche developed a 4-1BBL fusion protein targeting fibroblast activation protein (FAP) with Fc region mutations to abrogate FcγR binding but maintain favorable PK profile. The in vitro functional tests suggested that, only in the co-presence of anti-CD3 signal and FAP-expressing cells, FAP-4-1BBL can increase T cell activation and proliferation. Furthermore, preclinical studies showed that FAP-4-1BBL cannot inhibit tumor growth by itself. The combined treatment with relevant T cell-redirecting bispecific antibodies (TRBAs) or immune checkpoint inhibitor, such as anti-PD-L1, can efficiently inhibit tumor growth without prominent liver toxicity [93]. Recently, at the 34th SITC annual meeting, Pieris Pharmaceuticals reported the preliminary results of the Phase I study of PRS-343 (Her2 × 4-1BB) in patients with Her2+ malignancies. Among the 18 patients who received active doses of PRS-343, 2 patients reached partial response, and 8 patients had stable diseases. This is the first 4-1BB agonistic treatment reported with promising efficacy as well as good safety profile.

The tumor site localization strategy has also been exploited to selectively activate other co-stimulatory receptors, such as OX40, CD27, CD28, CD40, and ICOS, as well as to selectively inhibit co-inhibitory receptors, including CTLA-4 and PD-1. There are 10 bsAbs utilizing this strategy under active development at clinical and 19 at preclinical stages, which reflects the growing interests in this area (Table 2).

**Modulate TME.** To evade the immune surveillance, tumor cells can commonly influence the microenvironment around them by expressing immunosuppressive molecules, such as TGFβ and CD73, and by recruiting or promoting the differentiation of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs). A few bsAbs and bifunctional proteins are developed to overcome immunosuppressive TME, such as bintrafusp alfa, an anti-PD-L1, and TGFβRII fusion protein. TGFβ is a pleiotropic cytokine and plays dual functions in cancer progression. Though TGFβ suppresses tumor progression at tumor initiation stage, at later stages, TGFβ facilitates tumor progression and metastasis and has been suggested to contribute to resistance to anti-PD-1 and chemotherapy. Bintrafusp alfa (aka M7824) has the TGFβRII extracellular domain fused to the C-terminal of avelumab [94]. In preclinical studies, M7824 exhibited strong antitumor activity and significantly decreased the incidence of metastasis in mouse tumor models. In clinical tests, M7824 displayed acceptable safety profile and encouraging clinical efficacy in patients with heavily pretreated advanced solid tumors [95]. Other strategies targeting TME, including CD73 × TGFβ, EGFR × TGFβ, and CCR2 × CSF1R, are also under development at early clinical stage or preclinical stage (Table 3).

**Target cell depletion.** The last group represents the majority of the bsAb programs (clinical, 60/99; preclinical, 99/153) to promote the target cell depletion by different mechanisms (Table 4). According to the MOAs, this group can be further divided into subgroups, including cytotoxic effector engagement, Fc effector function (ADCC, ADCP, complement-dependent cytotoxicity [CDC]), enhanced phagocytosis, enhanced apoptosis, and drug conjugation.

Cytotoxic effector engagement is the largest subgroup in this category. Two out of the three launched bsAbs, catumaxomab, and blinatumomab are in this subgroup. Catumaxomab contains the antigen-binding sites for CD3 on the T cells and EpCAM on the cancer cells [96]. It was first authorized for market by the EMA in 2009 for the treatment of malignant ascites [97], but was withdrawn in 2017 due to commercial reasons. On the other hand, blinatumomab targeting CD3 and CD19 has shown impressive clinical results since launched in 2014 [98, 99].

T cells identify the target cells by recognizing the peptides presented by the major histocompatibility complex (MHC) through TCR. Based on the dynamic segregation model, the interaction of TCR with cognate peptide/MHC complex (pMHCs) brings the T cells and target cells in close proximity (∼14 nm) and results in TCR clustering at the center of immune synapse (IS) and exclusion of the large inhibitory tyrosine phosphatases from this region [100]. Following TCR clustering, cytolytic granules move toward the center supramolecular activation cluster (cSMAC) and release perforin and granzymes into the target cells. Once the target cells undergo cell death, the T cells quickly detach from the dying cells and move to the next target cell [100].

TRBAs are a group of bsAbs that can simultaneously target a component of the TCR complex (most commonly CD3ε) on a T cell and a target on the tumor cell surface [101–103]. By this approach, TRBAs promote IS formation between T cells and cancer cells independent of ligation of TCR with pMHCs, leading to T cell activation and killing of the tumor cells [104–107]. Due to the clinical success of blinatumomab, the development of TRBAs has gained substantial attention with 51 programs are at clinical and 66 at preclinical stages. Molecules in different formats are under evaluation to prove whether they can deliver the proposed biological function or have therapeutic window. For examples, BiTE and half-life extended BiTE are used by Amgen; DART and DART-Fc format are evaluated by MacroGenics; common light chain format is under investigation by Regeneron; and DuoBody format
is under development for multiple projects by Genmab and Janssen, Glenmark’s BEAT platform, Xencor’s XmAb platform, Aptevo’s ADAPTIR platform, and Teneo-Bio’s unique anti-CD3 platform are also under active exploration. Recently, IGM Biosciences announced the initiation of FIH Phase I clinical trial of IGM-2323, an IgM-based CD20 × CD3 TRBA. Unlike other formats, containing only 1 or 2 binding units for the tumor-associated antigen (TAA), the IGM-2323 contains 10 binding units for CD20. It is hypothesized that the higher avidity to CD20 of IGM-2323 may provide an advantage to treat CD20+ tumor cells over other formats. Moreover, the IgM-based TRBAs can more efficiently mediate CDC than IgG antibody. However, whether the IgM-based TRBAs can deliver superior efficacy to other formats still needs to be confirmed in the clinical studies.

Approaches to engage effector cell populations other than conventional αβ T cells, such as CD8 T cells, γδ T cells, NK cells, and iNKT cells, have also been explored and were reviewed by Ellerman recently [108]. The γδ T cells represent 10% of the total T lymphocyte population in circulating blood. Unlike conventional T cells, γδ T cells recognize stressed and malignant cells independent of MHC molecules, and their activation does not require costimulatory signals [109]. Besides strong cytotoxic activity, one unique property of activated γδ T cells is that they can cross-present tumor antigens to enhance CD8 T cell response [110]. A few strategies to improve the antitumor activity of the γδ T cells have been explored at preclinical and early clinical stages [111]. A selective Vγ9Vδ2 T cell engager (Her2 × Vγ9) showed superior activity in inducing Vγ9Vδ2 T cell-mediated tumor cell lysis than Her2 × CD3 TRBAs in vitro and exhibited antitumor activity in combination with IL-2 and activated γδ T cells adoptive transfer treatments in the PancTu-1 xenograft mouse model [112].

It has been argued that NK cell engagement may have better safety profile over T cell engagement therapies, while providing similar levels of clinical efficacy. CD16 is the most commonly used target for engaging NK cells. Results published by Affimed have suggested that NK engagers may induce efficient target cell killing with lower cytokine release risk, when compared to CD3 T cell engagers [113]. Early clinical results reported at 60th ASH meeting and 15th ICML meeting had shown that AFM-13 (CD16 × CD30) was well tolerated and efficacious when administered alone or in combination with pembrolizumab [114, 115]. The definitive clinical benefit of NK cell engagement still remains to be demonstrated in ongoing clinical studies. Other NK cell-activating receptors that are considered to have distinct advantages to overcome certain deficiencies in TME, such as NKG2D, NKp30, and NKp46, are under preclinical evaluation [116]. Additionally, strategies that redirecting iNKT cells by using CD1d extracellular domain fusion protein is also at early research stage [117].

Along with the growing depth of knowledge in Fc effector function, several approaches have been adopted for bsAbs, including mutations in the Fc region to enhance the FcγR binding, and afucosylation. Genmab has established a HexaBody platform, which contains mutations (E430G) in the IgG1 Fc region to enhance hexameriation upon antigen engagement and thereby enhance the CDC effect. By combining the HexaBody and DuoBody platforms, Genmab has developed a DuoHexaBody anti-CD37 biparatopic antibody. In ex vivo CDC assays using samples isolated from lymphoma patients, the DuoHexaBody anti-CD37 biparatopic antibody showed more potent tumor cell lysis, as compared to the control anti-CD20 antibodies, rituximab, ofatumumab, and obinutuzumab [118].

CD47-SIRPα signaling plays an inhibitory effect on the phagocytosis by phagocytes, such as macrophages. Tumor cells overexpress CD47 on the cell surface to escape the elimination by phagocytes. Antibodies against CD47 and SIRPα have been developed to interrupt CD47-SIRPα signaling. The combined treatment of a CD47 antagonist, Hu5F9-G4, with rituximab showed promising therapeutic efficacy in patients with non-Hodgkin lymphoma (NHL) [119]. By taking the advantage of the CD47 antagonist, a series of bsAbs using anti-CD47 as one moiety to enhance the phagocytosis to the cancer cells have been developed. Though there is only 1 program at clinical Phase I (NI-1701, CD19 × CD47), 14 programs are undergoing active development at preclinical phase with CD47 coupled with different tumor-associated antigens (Table 4), suggesting there is substantial growing interests in this area. Recently, the results published by Hatterer et al. have suggested that in addition to the enhancement of phagocytosis, the co-engagement of CD47 and CD19 by NI-1701 can prevent the colocalization of CD19 to BCR cluster during B cell activation, therefore inhibiting activated B cell proliferation [120].

Unlike previously mentioned bispecific programs, which all rely on the cytotoxic function of the effector cells or the complement system, two preclinical programs are focusing on actuating the apoptotic process of the cancer cells by activating the apoptotic receptors. BI and Promethera generated bsAbs targeting CDH17 × TRAILR2 (BI-905711) and CD20 × CD95 (Novotarg), respectively. According to the report published by BI on 2019 AACR Annual Meeting, BI-905711 induced TRAILR2 clustering on a CDH17-dependent manner and selectively triggered the apoptosis of CDH17 expressing tumor cells. BI-905711 also demonstrated significant antitumor activity in multiple colorectal cancer xenograft models [121].

Lastly, there are a couple of bsAbs that are being used to deliver toxin into cells that are positive for either or both targets based on the particular design of each molecule. For example, Regeneron is working on APLP2 × Her2 bispecific antibody-drug conjugate (ADC). Amyloid precursor-like protein 2 (APLP2) has been suggested to be involved in increased tumor cell proliferation and migration, and aberrant APLP2 expression was observed in multiple types of cancers, such as breast cancer [122]. Though APLP2 is an internalizing receptor, due to its ubiquitous expression and the presence of secreted form, APLP2-ADC is not an ideal target for ADC. Trastuzumab emtansine (T-DM1) has shown potent efficacy in cancer cells with high level of Her2 expression, but has little effect on cells with mid to low levels of Her2 expression. To improve the therapeutic efficacy of Her2 ADC, Regeneron developed the Her2 × APLP2-DM1. The bsAb binds to Her2-positive cells with the high-
affinity Her2 binding arm and then bridges to the cell surface APLP2 with the low-affinity APLP2 binding arm, which promotes rapid antibody internalization, lysosomal trafficking, and tumor cell killing [123].

**Bispecific antibodies for inflammatory conditions**

Autoimmune disease is the second largest area for bsAbs’ applications, with 10 clinical programs and 12 preclinical programs ongoing. Most of these programs are aiming to block the function of multiple pro-inflammatory cytokines by combining the neutralizing antibodies into one molecule entity, such as IL-1α × IL-1β, IL-17 × IL-13, IL-4 × IL-13, and BAFF × IL-17 (Table 5).

In immune cells, when it is in-cis coupled with an activating receptor, the inhibitory receptor can play a dominant negative role by diminishing the transduction of the active signal [124]. It has been shown that MGD-010 targeting CD79B, one component of the BCR complex and the inhibitory receptor CD32B, can decrease B cell response without depleting the B cells in healthy donors [125]. Ono is developing ONO-4685 (CD3 × PD-1) to turn down the T cell responses in autoimmune diseases. However, it is still not clear whether the effect of ONO-4685 is dependent on the in-cis engagement of CD3 and PD-1 to block the T cell activation or by in-trans interaction to deplete PD-1 expressing activated T cells.

**Bispecific antibodies for other conditions**

**Hemophilia A.** Hemophilia A is another successful example in the application of bsAbs, with the approval of emicizumab in 2017 as a landmark event. Emicizumab bridges factor IXa and X in spatially appropriate positions to facilitate the factor IXa-catalyzed factor X activation, which is usually mediated by factor VIII in healthy individuals, but is deficient in patients with hemophilia A. Though the etiology of hemophilia A has been well understood for a long period of time, the treatment options are still limited. Recombinant factor VIII and human plasma-derived factor concentrates are the commonly used practices for hemophilia A. However, the short half-life of factor VIII and development of anti-drug antibody (ADA) remains the major challenges for factor replacement therapy [126]. Emicizumab was intentionally designed to function as factor VIII with prolonged plasma half-life [127, 128]. Clinical results in hemophilia A patients with factor VIII inhibitor showed that the weekly subcutaneous (SC) treatment of emicizumab significantly reduced the frequency of bleeding episodes with no detectable anti-drug antibody [129]. Based on its promising efficacy and superior regimen schedule, emicizumab was initially launched in the USA for hemophilia A patients with factor VIII inhibitor in 2017; and then its usage was quickly expanded to patient without factor VIII inhibitor and was launched in EU and Japan in 2018. Similar programs are under preclinical development by Kymab and Shire.

**Ocular.** Excessive neovascularization, bleed, and fluid leakage from the abnormal blood vessels result in rapid vision loss or even blindness in patients with wet form age-related macular degeneration (AMD). Anti-angiogenesis treatment, such as Lucentis, Eylea, and Beovu, has been approved for treatment of this condition and has shown significant improvement in visual acuity and prevention of vision loss. Though over 90% of the patients can benefit from the treatment (i.e., maintain vision), eventually these patients become resistant to the treatment. New therapies are needed to further improve the therapeutic efficacy. As we mentioned above, several bsAbs have been developed to block the process of angiogenesis for cancer indications. Similar strategy has also been exploited for the treatment of wet AMD and diabetic macular edema (DME). Roche’s faricimab is an Ig-like bsAb targeting VEGF and ANGPT2 using CrossMab technology. During clinical tests, faricimab has shown superior efficacy and safety in patients with DME, as compared to Lucentis [130]. Phase III studies to evaluate faricimab’s therapeutic efficacy are initiated in early 2019; and the filing for BLA is expected in 2021.

**Neurology.** Despite the tremendous efforts in developing biological therapeutics for neurodegeneration diseases, effective treatment remains elusive. One of the obstacles in developing biological drugs for neurological disease is to effectively deliver the large molecule into the central neuron system. “Trojan horse” bsAb has one binding specificity responsible for the transportation of the antibody to the location that otherwise cannot be reached naturally, whereas the other binding specificity fulfills its function. By using this approach, a group of bsAbs have been developed to cross the blood-brain barrier (BBB). These antibodies usually have one binding arm recognizing the receptors in the receptor-mediated transcytosis system, such as insulin receptor, transferrin receptor, and lipoprotein transport receptors [131], and the other arm targeting the pathogenic molecules (Table 6). Bisfunctional fusion proteins or antibody-drug conjugates are also under active development as therapeutic drugs or diagnostic reagents for central nervous system diseases, but they are not under the scope of this review.

**Infectious diseases.** Due to the high frequency of escape mutations and development of drug resistance to single-agent treatment, combinatory treatment with mixture of mAbs or by bsAbs to broaden the protection spectrum and to decrease the chance to establish drug resistance is being developed to fight against infections. MEDI3902 was originally designed to achieve broader protection against _Pseudomonas aeruginosa_ by combining two clinically proven anti-PcrV and anti-Psl antibodies into one molecule. Psl and PcrV are present in ~90% of the _P. aeruginosa_ clinical isolates, respectively. Theoretically, the bsAb targeting Psl and PcrV simultaneously can protect the host from the infection of ~97–100% of the isolates, which express either or both targets. Surprisingly, when compared to the mixture of the parental antibodies in preclinical studies, MEDI3902 showed enhanced efficacy. By further dissecting the MOA, it was found that the format of MEDI3902 rendered a high-avidity low-affinity binding to Psl, which led to the accumulation of MEDI3902 around the bacterium and more efficient blocking of PcrV-mediated cytotoxicity [132].
The “Trojan horse” strategy is also employed by some bsAbs with elegant design for infectious diseases treatment. During filoviruses (e.g., Ebola virus) infection, the membrane envelope glycoprotein (GP) is responsible for the cell attachment and membrane fusion. A unique feature about the GP of filoviruses is that it first binds to the receptor on the cell surface which induces the internalization of the virus particle, and then in the late endosome, GP is cleaved to expose the highly conserved receptor-binding site (RBS) for Niemann-Pick C1 (NPC1), which mediates the membrane fusion and cell entry [133]. Therefore, to provide broad protection against filoviruses, bsAbs were designed to block the intracellular GPCL-NPC1 interaction by coupling the GPCL-NPC1 blocking arm with a delivering arm targeting a broadly conserved epitope in uncleaved GP. The delivering arm binds to the virus particles and goes into the endosome together with the virus, where the blocking arm functions to abrogate the GPCL-NPC1 interaction when it is exposed and prevents the viral entry [134].

Diabetes. Fibroblast growth factor 21 (FGF21) plays key roles in stimulating metabolism and has shown some preliminary clinical benefits in obese patients with diabetes. However, the poor PK profile and potential adverse effects associated with long-term usage of recombinant FGF21 limit its usage. RG7992 (FGFR1 × KLB) was therefore designed to mimic the function of FGF21 but selectively activate FGFR1/KLB complex in the liver, adipose, and pancreas tissues, where KLB is present, to avoid safety concern associated with broad FGFRs activation, but still be able to provide clinical benefit in obesity and diabetes [135].

MATCH BIOLOGY WITH AN OPTIMAL BISPECIFIC FORMAT

As discussed in the early section, format diversity is essential to serve the plethora of applications of bsAbs defined by TPPs. Variances in affinity, valency, epitope, and geometry of their binding domains, linkers, as well as in size- and Fc-mediated distribution and pharmacokinetic properties to fulfill a particular clinical application define a bsAb format. In practice, many variances or attributes for selecting an optimal format are intertwined and must be addressed for selecting the right molecule. Therefore, we will discuss these attributes below.

Antigen-binding affinity and valency

Affinity: Even though one of the advantages of using antibody-based therapeutics is that they may interact with their antigens with substantially high affinities, higher affinity does not always translate into better efficacy. Unlike antagonistic molecule, whose potency is usually associated with its affinity, agonistic molecule is more difficult to predict and to optimize its potency by increasing the binding affinity. Based on different modes the receptor uses for activation, different binding kinetics of the agonistic bsAb to reach optimal receptor activation are required. For receptors depending on clustering to activate, fast-on fast-off binding kinetics is preferred to ensure efficient recruitment of receptors [136, 137]. On the contrary, for receptors activated by ligand binding-induced conformational change, the slow off binding kinetics would endorse more durable activating efficacy [138]. Furthermore, there are evidences that the affinity to CD3 may significantly affect the function and safety profile for TRBAs. It has been suggested that T cells require lower threshold for mediating cytokotoxic killing than for cytokine production perhaps due to different number of ITAM motifs of TCR complex being phosphorylated, it may be possible to dissociate TRBAs’ potency from toxicity by modulating the CD3 affinity of the bsAbs. As shown by Leong et al., by lowering the affinity to CD3, the CD3 × CLL1 bsAb with low affinity to CD3 exhibited better safety profile and retained equivalent in vivo efficacy, as compared to the ones with high affinity to CD3 [139] when net impact on T cell activation, receptor internalization, and PK all combined. Similar results were also shown by Zuch de Zafra et al. By comparing a series of CD38 × CD3 bsAbs with different affinities to CD3, they found that lowering the affinity to CD3 can dramatically decrease the cytokine release, but still maintain potency in mediating cytokotoxic killing [140]. In November 2019, AMG-424, the final lead from the aforementioned study, was granted with orphan drug designation for multiple myeloma by the FDA.

As for the affinities of TRBAs to TAAs, due to the different expression profile of the TAAs in normal tissues versus in tumors, and the tolerability and the ability of regeneration of TAA-positive cell populations in normal tissues, the TRBAs targeting different TAAs may require different binding kinetics. For low-expression, tumor-specific antigens, a TRBA with high affinity to the antigen would be required to elicit efficient tumor cell killing. However, for TAA with low expression on essential normal tissues/ organs, to spare the normal cells and avoid on-target off-tumor toxicity, low-affinity high-avidity TRBAs would be preferred, which can be achieved by modulating the valency (see below).

Moreover, for a bsAb, difference in affinities of two different antigen-binding specificities may determine which arm drives tissue distribution, tissue penetration, and retention of a therapeutic molecule at the site of MOA. For examples, high affinity to TAA and low affinity to CD3 may enable the preferential binding of TRBAs to the target cells and implement serial killing of the target cells by a single T cell [141]; and as mentioned above, APLP2 × Her2 bispecific ADC with high affinity to Her2 and low affinity to APLP2 preferentially binds to Her2-positive cells and then bridges APLP2 on the cell surface to mediate efficient endocytosis to avoid the toxicity associated with the pan expression of APLP2.

For BBB crossing bsAbs, along with other considerations, careful selection of the transport receptor and selection of a molecule with appropriate binding kinetics to the transport receptor is critical for success of this strategy. As reported by the scientists from Genentech, to ensure the effectiveness of the transcytosis, the “Trojan horse” antibody using the TfR pathway needs to have low affinity
to TIR [142]. Later, another study by the University of Wisconsin-Madison showed that TIR bsAb with high binding affinity to TIR at pH 7.4 but low affinity at pH 5.5 can effectively release the bsAbs from BBB into the brain and avoid the degradation of bsAb in the endosome [143].

Valency. The valency for each target can dramatically affect the function of the bsAbs. For the TRBAs, monovalency of anti-CD3 arms may help to avoid non-specific activation of the T cells without engagement of tumor cells, as shown by Bardwell et al. [144]. Interestingly, Y-mAb and Abpro have CD3 scFv fused to the C-terminus of the light chain. Even though the format ends up with two binding sites for CD3, both companies claimed that this format was actually functional monovalent toward CD3. Additionally, Aptevo and Affimed also developed TRBAs using bivalency to CD3. Preclinical evidence has suggested that the adoption of the ADAPTIR format can induce potent T cell activation and target cell killing, but low levels of cytokine release [145]. AFM-11 (CD19 × CD3) also showed more potent T cell activation than BiTE control and strict CD19-dependent T cell activation preclinically [146]. However, due to one death and two life-threatening events in clinical trial, AFM-11 was placed on clinical hold.

The valence for the TAA may vary based on the properties of the TAA, such as tumor specificity, antigen size, expression level on the tumor versus normal tissue, and the tolerance of complete elimination of TAA-positive cells. In the case of some types of hematopoietic tumors, the depletion of both normal and malignant cells expressing TAA, such as CD19 and/or CD20, can be tolerated. However, for most of the other TAA, the expression levels may be low on normal tissues, but the killing of these low-expression normal cells can lead to deleterious consequences. To distinguish the target high tumor cells from the target low normal cells, RG7802 (CEA × CD3) was optimized to have low-affinity high-avidity 2 + 1 format in appropriate geometry to facilitate the selection of CEA high cells with a threshold of ~10 000 CEA-binding sites/cell [105].

Based on the lessons learned from the initial mAb development for cMET treatment, bivalency to cMET may elicit agonistic, instead of antagonistic, effect resulting from the mAb-mediated dimerization of cMET. Though monovalent binding to cMET can function as an antagonist, it can only block the HGF-mediated cMET activation. Later, Wang et al. demonstrated that ABT-700, a truly antagonistic mAb against cMET, can bind to a unique epitope on cMET. The bivalency to cMET of ABT-700 and stringent hinge region was essential to inhibit both HGF-dependent and HGF-independent activation of cMET and induce cMET downregulation [147]. Interestingly, half of the cMET bsAb programs are using monovalency against cMET to avoid agonistic effect, while the other half choose bivalency. EMB-01 (EGFR × cMET) has two binding sites for cMET, and has no obvious cMET activation in the absence of ligands. Furthermore, it can effectively induce EGFR and cMET degradation, therefore preventing the cMET activation [62].

Epitope, geometry, and distance between different antigen-binding domains

Epitope. In respect of antagonistic bsAb, the binding epitope of the corresponding binding units are required to prevent the receptor/ligand engagement, or the receptor signal complex formation, or any step that is crucial for the initiation or passage of signaling cascade into the cells to play its biological function.

In general, the receptor-binding epitope for agonistic molecules is not as predictive as it is for antagonistic molecules. However, there is evidence to suggest that the binding epitopes do contribute significantly to the bsAb efficacy. It was found that anti-CD3 binding arms recognizing different epitopes on CD3-activated T cell differently. TeneoBio, therefore, identified a dozen of anti-CD3 antibodies with different binding epitopes and different binding kinetics to CD3 molecules to disassociate the capabilities of TRBAs in inducing cytotoxic killing from promoting cytokine production post T cell activation. They identified a clone (F2B) that recognizes a unique epitope on CD3δε, but not CD3 γε, at a low affinity (34 nM). By comparing to another clone (F1F), which binds to both CD3δε and CD3 γε with high affinity (<1 pM), they found that BCMA × CD3 bsAb using F2B arm (CD3_F2BxBCMA) can induce moderate levels of cell killing but very weak cytokine production, as compared to the one using F1F arm (CD3_F1FxBCMA) in vitro. Moreover, the in vivo efficacy study showed that CD3_F2BxBCMA exhibited antitumor activity in a wide dose range (0.01–10 μg), while CD3_F1FxBCMA completely lost its therapeutic efficacy at the high dose (10 μg) [148]. As we mentioned above, to effectively redirect T cell killing, the TRBAs must be able to induce the IS formation between the T cells and target tumor cells. Besides the format of the TRBAs, the tumor antigen selection, the size of the antigen, antigen surface density, as well as the distance between the TRBAs binding epitope to target cell membrane, all can influence the formation of the IS. Comparing to large antigens or antigens with protruding structure, the small antigens or antigens with structure close to the cell membrane can more effectively promote the IS formation [106]. When the selected tumor antigen is large in size, such as melanoma chondroitin sulfate proteoglycan (MCSP) [149] and FcRH5 [150], the membrane-proximal epitope is desired. For cell surface targets that can be shed into the bloodstream, to avoid antigen sink, the bsAbs should recognize the membrane-bound but not the soluble form of the antigen [151].

Geometry. Besides the distance between the epitope to the target cell membrane, the distance between the two targets engaged by TRBAs also plays a crucial role in determining whether it can effectively promote IS formation and T cell activation. Considering the distances between the TAA and CD3 epitopes to target cell and T cell, respectively, the format of the TRBAs needs to bring TAA and CD3 to a close proximity much less than 14 nm. Moreover, the whole molecule has to be able to physically fit into the small junction between the two cells in a density to effec-
tively form a cluster with several engaged target pairs to initiate TCR signaling. Despite its short serum half-life, the small size of BiTE format with two binding units located in opposite sides is extremely potent in redirecting T cell cytotoxicity by inducing serial killing of tumor cells at an effector-to-target ratio as low as 1:5 [141]. In another case, Aptevo fused the scFvs against the TAA and CD3 at the N- and C-terminus of Fe (scFv + scFv with Fc, 2 + 2), which ended up with longer distances between the two binding domains. The in vitro studies showed that this molecule had more potent target cell killing, but less cytokine release, as compared to the BiTE format [145]. Unfortunately, the clinical development for this molecule was discontinued due to high frequency of anti-drug antibody development.

The same situation also applies to T cell co-stimulatory and co-inhibitory receptors, which co-cluster with TCR during IS formation and regulate T cell activation. PD-1 and PD-L1 interaction leads to the accumulation of PD-1 microclusters at cSMAC and destabilizes the IS. When the extracellular domain of PD-1 was elongated by inserting extra Ig domains, the inhibitory role of PD-1 decreased along with the increase of the number of Ig domains inserted [152]. Though current anti-PD-1 molecules all block the PD-1 signaling by inhibiting the PD-1/PD-L1 interaction, in theory, the designs that can prevent the PD-1 colocalization to cSMAC should also be able to diminish the inhibitory role of PD-1 in T cell responses. On the contrary, bsAbs to activate the co-stimulatory receptor such as 4-1BB must exert its function at the site of IS [93]; therefore, a format that can meet these criteria is necessary. As reported by Pieris, the geometry of the 4-1BB anticalin attachment significantly affected the function of the Her2 × 4-1BB bispecific anticalins. PRS-343 with 4-1BB anticalin attached at the C-terminus of the heavy chain showed the most effective T cell activation, as compared to other formats. One possible explanation is that the binding sites for Her2 and 4-1BB are approximately 15 nm apart, which is close to the distance of the IS. However, after measuring the distances from the binding epitopes to the cell membrane, the distance between the target cell and the effector cell might be much longer than 15 nm. On the other hand, ND-021 (PD-L1 × 4-1BB × HSA) is an Fc-lacking scFv-VHH-based molecule. With its small size and flexible structure, it may have better potential in colocalizing at cSMAC and enhance TCR signaling. It will be interesting to see how it will perform in the clinical trials.

Cases are also shown in bsAbs programs developed for other conditions. For example, when the scientists at MedImmune tested their Psl × PcrV bsAbs, they examined several different formats with varying intramolecular distances between the two binding components. After comparison of these formats in both in vitro and in vivo efficacy studies, BiS4aPa, with an intermediate distance, exhibited the most effective protection against P. aeruginosa infection and therefore was selected as the final therapeutic candidate format [132].

**Linker design**

As reviewed by Brinkmann and Kontermann, various connecting linkers have been explored [3]. Similar to the hinge region of the IgG subclass, the length, flexibility, and amino acid composition of the linkers used to connect the building blocks (scFv, Fab, etc.) may determine the correct formation, functionality, and developability of the resulting bispecific molecules, as shown by Le Gall et al. [153] and DiGiammarino et al. [154].

**Size**

The bsAbs have made significant impact on hematologic malignancy treatments. However, the therapeutic benefits delivered by bsAb for solid tumor are still waiting to be unveiled. One of the concerns using bsAbs for solid tumor treatment is how to increase the drug tumor penetration and accumulation. Though molecules with smaller size would have better chance entering the tumor site by increased tumor penetration, the molecules with size smaller than the threshold of renal clearance of proteins are rapidly cleared from the blood and therefore have decreased flux into the tumor [155]. Using a compartmental model, Schmidt and Wittrup predicted that molecules with the size of 150 kDa would have the best tumor localization, whereas molecules with the size of 25 kDa would have the worst tumor uptake [156]. However, due to their large size, molecules at the size of ~150 kDa have decreased extravasation and normally take days to reach maximum tumor uptake. On the other hand, molecules of smaller size reach the maximum tumor uptake within a short period of time. The fast tumor penetration and systemic clearance of small-sized molecule therefore lead to high tumor/blood localization ratio, which is preferred for some applications, such as imaging [157], as well as safety management to decrease the systemic drug exposure-associated toxicity.

To improve the serum half-life, while still retaining the fast extravasation property, Harpoon developed the TriTAC platform, which targets TAA, CD3, as well as human albumin for extended half-life with a total size of ~50 kDa. It is believed that with its improved drug exposure and small size, TriTAC would enable faster and better tumor penetration, compared to large-sized bsAbs.

**Fc region**

The Fc region can substantially influence the bsAbs’ function. It was found that the properties of IgG subclass hinge region, such as length, sequences, flexibility, and disulfide bond structures, can influence the variable region presentation and thereby affect the functionality of an antibody [158]. While it is not always desired, the format with Fc can prolong the bsAb serum half-life through FcRn-mediated recycling and may provide Fc effector function through the interaction with FcγRs.

**IgG subclass.** Recently, Kapelski et al. reported the influence of the IgG subclass on TRBAs. They found that due to its short and rigid hinge region, IgG2 cannot effectively promote the IS formation. However, by replacing the hinge region of the IgG2 with the hinge region of IgG4 or IgG1, the function of IgG2 chimeric bsAb can effectively induce IS formation and redirect T cell killing [159].
Similarly, the Fc region also showed significant influence on the factor VIII-mimetic activity of emicizumab. After comparison between different IgG subclasses, interchain disulfide bonds, and mutations in hinge region and CH2 domain, IgG4 was selected as it presented with the most potent factor VIII-mimetic activity [160].

**Fc effector function.** As mentioned above, several strategies have been developed to enhance the binding between Fc and FcyRs to increase the Fc effector function. This could be important for bsAbs against TAAs for effective killing tumor cells or for bsAbs against infectious agents for pathogen uptake and clearance. However, the Fc effector function and FcyR binding are usually abrogated for TRBAs and some other agonistic bsAbs to avoid the FcyR-mediated cross-linking, which may cause non-specific activation of T cells and the targeted receptors, respectively. Advances in Fc engineering allow tailored modification of Fc effector functions for specific need. For example, Xencor developed a series of TRBAs using the XmAb platforms, including AMG-424 (CD38 × CD3, Fab + scFv with Fc, 1 + 1), and used a combination of mutations (E233P/L234V/L235A/G236del/S267K) to completely eliminate the binding of IgG1 Fc to Fcy Rs [55].

Because IgG4 only binds to FcyR1 with high affinity and mediates weaker effector function than IgG1, it is commonly used for antagonistic antibodies targeting immune cells, to avoid Fc effector function-mediated cell elimination. However, the research by Zhang et al. showed that the anti-PD-1/IgG4 antibody can induce the phagocytosis of PD-1+ T cells by activating FcyR1+ macrophages. By introducing five additional mutations (E233P/F234VL235A/D265A/R409K), BGB-A317 showed no binding to FcyR1, and more efficient preclinical antitumor activity, as compared to the anti-PD-1/IgG4 control [161]. The recently reported results of the pivotal study of BGB-A317 also exhibited its superior antitumor efficacy in patients with relapsed/refractory classical Hodgkin lymphoma, with an overall response rate (ORR) of 87% and 63% complete response rate (CRR).

As we discussed above, the format contains many components that can be tweaked, their final impact on pharmacological properties of a bsAb is intertwined, and here we only mentioned some of them. The fine-tuned parts work in concert with each other to determine the success of bsAbs. To obtain the optimal therapeutic candidate, the selection of any component in the final format should be carefully evaluated for specific target pairs; and the matched format will not only facilitate the bsAbs to elicit biological function but also may enable a molecule for further product development, which otherwise may not be suitable for clinical application.

**SELECT A RIGHT MOLECULE TO MEET BOTH FUNCTION AND DEVELOPABILITY REQUIREMENT**

As illustrated in the early section, the six criteria critical for clinical development and commercial manufacturing (Fig. 1) define a good bsAb. Identification of a good therapeutic bispecific molecule usually requires starting with good therapeutic and molecular design defined by MPP that is developed based on TPP, followed by vigorous in vitro and in vivo screening and characterizations. Below we will discuss these six criteria and how they can have significant impact on the outcome of the resulting bsAbs: physiochemical properties, manufacturability, immunogenicity, PK/PD property, and, most importantly, efficacy and safety.

**Physiochemical properties and manufacturability.** As aforementioned, many strategies have been explored to solve the CMC quality issues, such as mispairing, stability, aggregation, segmentation, solubility, viscosity, purification, etc. A good bispecific clinical candidate should (1) be easily expressed with high percentage of correctly assembled product in manufacturing scale; (2) display no significant aggregation or low percentage of aggregation that can be easily removed, as aggregation may affect the therapeutic efficacy and increase immunogenicity risk; (3) have good solubility, high stability, and low viscosity to meet drug product formulation needs for intended clinical dosage and route of administration; and (4) have low manufacturing cost for economical reason. The stringency of those requirements may differ based on various clinical applications. For instance, reconstituted lyophilized formulation for intravenous infusion (IV) is generally acceptable for oncology applications, while liquid formulation developed for SC administration may be preferred for most of autoimmune indications. For ocular disease, the high solubility, high stability, and low viscosity are imperative for a competitive product. With the advance of the bsAb technology, more and more reported bsAb formats can be expressed and purified with reasonable yield and meet the reasonable physiochemical properties for a given clinical application and are scalable for large-scale manufacture, although some of the formats do require significant CMC optimization and longer development timeline than the others.

**Immunogenicity.** Immunogenicity is one of the critical factors limiting clinical use of biological therapeutics, as the generation of ADA may lead to fast drug clearance, neutralization of therapeutic effect, and even severe adverse events in clinic. The duration of the ADA response can be categorized into transient and persistent ADAs. The persistent ADA requires the T cell help and commonly leads to more deleterious consequences. The nature and the levels of the ADA generated are influenced by both the patients’ physical conditions (autoimmune-prone vs. immunosuppressive, pre-existing ADAs, etc.) and the intrinsic properties of an antibody (i.e., sequences, impurities, format, MOAs, dosing regimens, etc.) [162]. For example, the cancer patients are usually immunosuppressive, while the patients with autoimmune diseases are prone to develop auto-reactive antibodies and ADAs. The antibodies that contain strong T cell epitopes have high risk to induce T cell-dependent persistent ADA. Compared to bsAbs that deplete B cells, the bsAbs that enhance immune system response may have a higher chance to induce ADA. And the bsAbs dosed by SC and intramuscular (IM) administration may be easier...
to be picked up by dendritic cells (DCs) and present bsAb-derived peptides to T cells, as compared to the ones given by IV infusion.

As reviewed by Davda et al. [163], using the approved mAb clinical results, they found that although both atezolizumab and durvalumab were Fc-engineered anti-PD-L1 mAbs, only atezolizumab showed higher rate of ADA, as compared to the other anti-PD(L)1 mAbs. The combination of the anti-PD(L)1 and anti-CTLA-4 could increase the rate of ADA; the ADA rates against nivolumab were increased from ~12% (monotherapy) to 24–38% (combo therapy with ipilimumab). Furthermore, the antibodies mediating B cell depletion usually had low ADA rates. As most of the reported bsAb formats are heavily engineered and with non-native Ig sequences introduced, it is very likely that the bsAbs have higher immunogenicity risk than regular mAbs. However, most of the bsAb programs are still at early clinical stages, and only very limited information and results are available to evaluate the immunogenicity issue for bsAbs. As mentioned above, Aptevo developed APVO-414 (PSMA

PD, described as what the drug does to the body, involves the target binding and the following effect. The PK/PD profiles play an important role in effecting the drug efficacy and safety and therefore are critical for the development of bsAbs. Many factors of the bsAbs can influence the PK profiles, including molecule format, size, physicochemical properties, FcγR binding, as well as target binding affinity. For example, Harpoon is developing a novel protease-activated T cell engager platform, ProTriTAC based on the aforementioned TriTAC platform. By modifying the non-CDR region, the anti-albumin SDA can bind and mask the anti-CD3 arm while maintaining its binding to albumin. Furthermore, a tumor-associated protease cleavage site is introduced to the linker between the anti-CD3 binding domain and anti-albumin SDA. In the circulation, the anti-albumin keeps anti-CD3 arm inactive and imparts the molecule long serum half-life. Once it enters into the TME, ProTriTACs are cleaved by tumor-associated proteases to lose the anti-albumin SDA and expose the anti-CD3 binding site to function. If the cleaved molecules enter into the circulation again, they will be rapidly cleared from the system due to its small size. By using this strategy, they developed a ProTriTAC targeting EGFR, which was not easy to be targeted by TRBAs due to its wide expression in the normal tissue.

As long serum half-life may increase tissue penetration and therapeutic efficacy, as well as require lower dosage and less frequent drug administration, sometimes, a longer serum half-life is preferred. IgGs and albumin are both abundant in plasma with long half-life due to the binding to FcRn, which rescues them from degradation in the endo/lysosomal compartment. Therefore, enhancing the Fc binding to FcRn, or by adding a HSA binding domain into the format (without Fc), is commonly used by bsAbs to improve the serum PK. Many mutations in the CH2-CH3 region have been tested to increase the Fc binding to FcRn, yet only the YTE and LS mutation combinations (YTE = M252Y/S254T/T256E; LS = M428L/N434S) have been clinically validated [165]. YTE mutations can increase the antibody serum half-life ~4-fold in humans, but also decrease the ADCC activity of the antibody. YRFC01LS containing the LS mutations also showed more than 4-fold increase in serum half-life in human [166]. Unlike YTE mutations, LS mutations have no impact on antibody’s ADCC activity. On the other hand, in some applications, when the prolonged half-life is undesired, mutations to decrease the Fc to FcRn binding can also be applied. Detailed methods in modulating FcRn binding to modify PK were reviewed by Leipold [167]. Though the effect of FcRn on influencing serum half-life has been well studied, it still remains controversial on how it affects the drug metabolism in other tissues, such as the brain and eyes. As matter of fact, Lucentis (Fab) and Eylea (Fc fusion protein) only showed slightly different ocular half-life in humans, suggesting FcRn binding may not play a major role in determining ocular half-life, while the molecular size may play some, but not determining roles on PK properties of molecules in retina.

Pharmacokinetic and pharmacodynamic properties. PK, described as what the body does to a drug, refers to the drug absorption, distribution, metabolism, and excretion.
surface antigens. As mentioned in the previous section, decreasing the target binding affinity, in some cases, can prolong the drug half-life and therefore improve the therapeutic efficacy. As shown by Leong et al., the relationship between CD3 affinity of the CD3 × CLL1 TRBA to its activity, PK and safety are quite complicated. During in vitro characterization, they found that the one with low affinity to CD3 (CLL1/CD3L) showed decreased potency, but had more favorable safety profiles, as compared to the one with high affinity to CD3 (CLL1/CD3H). More importantly, when they tested these molecules in vivo, they found that CLL1/CD3L had slower drug clearance (50%) and increased drug exposure, which led to more durable antitumor responses, as compared to CLL1/CD3H [139]. A similar case was also observed in an IL-15/RA × PD-1 bifunctional protein. The fusion protein was engineered to decrease the IL-15/RA potency, thereby decrease the antigen sink, and increase half-life. Several variants with decreased potency were generated and compared in vivo. As they predicted, the low potency variants showed dramatic half-life extension from 0.5 day (wild type) to 9 days [US20180118828].

Besides affecting the serum PK, target binding affinity may also influence the tumor/tissue distribution of the bsAbs. For example, the affinity of the bsAb to the tumor antigen can influence the tumor penetration. BsAbs with extremely high affinity to tumor antigen get stuck at the entrance and therefore have poor tumor penetration [168, 169]. While low-affinity bsAbs distribute further into the tumor, but bsAbs with small sizes may have decreased retention time in the tumor. The distribution of TRBAs for solid tumors, as predicted by Friedrich et al., may be significantly affected by the distribution of T cells, and modifying the affinity to CD3 or TAA may not be sufficient to accumulate TRBAs and T cells into the tumor [170]. Other methods may be used to affect the bsAb distribution inside the tumor tissue and include target selection, Fc, and utilizing of transcytosis [155].

The ultimate goal of all previously discussed strategies to modulate PK was to enhance the overall clinical efficacy and/or to minimize the toxicity of the therapeutic bsAbs. Similarly, improving the PD profiles can also be achieved by modifying the antigen-binding activity and by modifying the Fc-mediated effector function to further increase clinical potential of the bsAbs. Thus, the PK/PD profiles can be modified by adjusting multiple factors, while most of these factors are interdependent, which highlight the inherent challenges in therapeutic antibody design, and improving one property can sometimes affect the others. Therefore, we should bear in mind that due to the complexity of the MOAs of bsAbs, the PK/PD profiles may not be the same as we expected (hoped). Robust technologies and tools (both experimental and in silico) are critically needed to advance the understanding of structural determinants of the bsAbs that can impact the PK/PD properties and to guide the optimization of bsAbs.

Efficacy and safety. A reasonable efficacy/safety window is fundamental for a good clinical candidate; and PK/PD profiles, efficacy, and safety profiles commonly influence each other. It is common that the drug showing high potency in discovery stage tends to be selected as the therapeutic candidate. However, highly potent drug that induces toxicity at low dose leaves no or very limited therapeutic window, which may significantly hinder its clinical application. On the other hand, the drug with a reasonable potency but better safety profile may have wide therapeutic window, and the therapeutic efficacy may be improved by readily increasing the dose without inducing significant toxicity. Increasing drug exposure may be another way to enhance the efficacy and prolong the response duration, as we discussed above. However, increased systemic exposure may also increase the chance and the severity of adverse event. It is hard to predict which composition of the MPP can translate into an optimal TPP in clinical application.

For example, despite its extreme potency in eliminating the tumor cells, the life-threatening adverse effect associated with the treatment of blinatumomab, as well as short serum half-life, both significantly limit the application of blinatumomab [171]. To improve the therapeutic efficacy and prolong the serum half-life, Affimed developed AFM-11 (Fv + Fv, 2 + 2), a tetravalent CD19 × CD3 bsAb [146]. In vitro characterization studies showed that AFM-11 was more potent than BiTE molecule to elicit target cell killing. Though bivalent for CD3, AFM-11 showed stringent target-dependent activation of T cells. Using a NOD/SCID xenograft model, AFM-11 showed favorable PK profiles with preferential tumor localization over normal tissue and a half-life of ~20 h. In Phase I dose escalation study, AFM-11 was dosed by continuous infusion (Week 1, 0.7 ng/kg/wk to 130 ng/kg/wk; Week 2+, 2 ng/kg/wk to 400 ng/kg/wk). During the study, among the 14 patients who completed the dose limiting toxicity observation period, 3 patients showed complete response (CR), but 2 were transient and patients relapsed after cycle 2. Serum half-life was ranged from 7.14 to 10.6 h in four evaluable patients. Although no cytokine release syndrome (CRS) was observed, two Grade 3 neurotoxicity and one fatal event were recorded in the two highest dose groups. AFM-11 was placed on clinical hold, due to the severe adverse events.

TRBAs in formats containing Fc may have improved stability and manufacture profile, as well as prolonged serum half-life. The long-term drug exposure may provide improved efficacy and more flexible dosing strategy, but may be more difficult to handle if undesired effect is experienced. Regeneron developed REGN-1979 (Fab + Fab with Fc, 1 + 1), a CD20 × CD3 bsAb. In vitro assays showed that REGN-1979 can effectively and specifically mediate the killing of CD20⁺ cells. The preclinical pharmacology studies using cynomolgus monkeys showed that REGN-1979 can cause durable and deep B cell depletion with a serum half-life of ~14 days [31]. In June 2019, Regeneron reported the early-stage dose escalation trial results of REGN-1979: 93% ORR and 71% CRR in 14 patients with follicular lymphoma treated with REGN-1979 (5–320 mg); and 57% ORR in 7 patients with diffuse large B cell lymphoma (DLBCL) treated with REGN-1979 (80–160 mg), which were all CR. Among the total of 81 evaluable patients, 7% experienced Grade 3 or higher CRS, and at least 10% of patients experienced Grade 3 or higher
adverse event. The incidence and severity of CRS can be mitigated by optimized premedication. Recently, in 2019 ASH annual meeting, similar results were also reported for mosunetuzumab (CD20 × CD3, Roche) with ORR and CRR of 62.7 and 43.3%, respectively, in patients with slow-growing non-Hodgkin lymphoma. Both REGN-1979 and mosunetuzumab showed benefit to patients who had disease progressed post CAR-T therapies.

The comprehensive review regarding TRBAs published by Ellerman made a perfect case of how complex it can be to optimize a TRBA, and the change of a factor of the bsAb may influence multiple profiles of the molecule, and a molecule profile can be modulated by multiple factors. For example, to uncouple the capabilities of TRBAs to induce cytotoxic killing and cytokine production by the T cells, the TRBAs can be modified by (1) decreasing the affinity to CD3, as T cell cytotoxic killing requires a lower activation threshold; (2) using a different CD3 binding epitope, as based on the “permissive geometry” model, different binding epitope may lead to different CD3 conformational change and T cell signaling; and (3) switching to another format. In another case, to distinguish the antigen-overexpressing tumor cells and the low-expression normal cells, one can (1) decrease the binding affinity and use multivalency to the antigen and (2) increase the distance of the IS, by either choosing a membrane distal epitope on the antigen or using a format with longer distance between the two binding domain. From another aspect, decreasing the affinity to CD3 may diminish the target cell killing potency in vitro; it may also increase the PK profile and tumor accumulation which ends up with comparable or even improved in vivo efficacy and therapeutic window [108].

Another group of bsAbs that represents with challenges in leveraging the safety and efficacy is the agonistic bsAb targeting co-stimulatory receptors, such as 4-1BB. Recently, the results reported for PRS-343 showed first sign of hope for development anti-4-1BB treatment (see above). Numab developed ND-021, a monovalent trispecific antibody targeting PD-L1, 4-1BB, and HSA. The in vitro efficacy tests suggested that the ultrahigh affinity (2 × 10^{−12} M) to PD-L1 determined the potency of the molecule: binding to a distal epitope on 4-1BB can promote the 4-1BB clustering more effectively; and when the affinity to 4-1BB was way lower than the affinity to PD-L1, the effective dose range can be significantly extended. As compared to the combinations of mAbs, ND-021 showed superior activity in enhancing activated T cell responses. Due to the monovalency and lack of Fc region, ND-021 displayed strictly PD-L1-dependent 4-1BB activation and spared antigen-presenting cells from depletion. In in vivo efficacy tests, ND-021 showed higher antitumor activity than combined treatment with mAbs in mice. Most importantly, ND-021 did not induce liver toxicity, and systemic T cell activation in cynomolgus monkey posts a single-dose IV injection [172] although it remains elusive how this may translate into safety in humans. Currently, this program is at IND-enabling study stage, and we are looking forward to see its clinical results.

On the basis of the strong biological rationale, empowered by the carefully harmonized format, and with the meticulously selected binding units, bispecific molecules just finish the first step to its final success. A good bispecific clinical candidate not only needs to show promising therapeutic potential but also needs to have good physicochemical properties and scalable manufacturability. Furthermore, favorable PK properties and low immunogenicity are also critical to assure the success of the candidate. Besides all the above mentioned factors, the efficacy/safety ratio is one of the major determinants whether a bsAb moves into development stages in the end.

KEY CHALLENGES THE FIELD STILL FACING

Though bsAbs development has made significant progress and several strategies have been exploited to solve some of the challenges, many still remain. We would like to review these challenges in two categories: technical challenges and mechanistic or biology challenges.

Technical challenges

Discovery: Compared to mAbs, bsAbs display significant complexity in the research and development stages. Special testing systems are needed to characterize the potential therapeutic efficacy, toxicity, and PK/PD profiles of the bsAb therapeutic candidates, and many of these systems may be quite complicated, as compared to the systems used to evaluate mAbs.

For example, artificial cell line used to evaluate bsAb function needs to overexpress both targets and include both signaling pathways, and the generation of such cell line may have huge technical challenges. Also, the expression level and temporal order of the two targets on the artificial cell line may not reflect the disease situation in human. For primary cell-based efficacy tests, a specific population of cells may need to be isolated and cultured ex vivo to induce the expression of both targets, which makes the assays extremely time- and cost-consuming and low throughput. Furthermore, even though researchers try to mimic the real situation under which the bsAb plays its functional roles, the in vitro assay system cannot completely reflect the immune system, and therefore the effect of the bsAb cannot be accurately evaluated in vitro.

The selection of species and relevant disease model for efficacy, pharmacology, and toxicology studies can be complicated, with considerations for the properties of both targets, such as the cross-species specificity, the functionality of the bsAb, as well as the expression and function of the targets. Although, transgenic animals and animals grafted with human immune systems are developed for bsAbs without cross-species binding, it is still doubtful how closely these models can reflect the actual clinical conditions and how accurately they predict the therapeutic efficacy, safety risk, and PK/PD profiles of a bsAb.

CMC. With the advanced protein engineering technology and elegantly designed bispecific formats, the physicochemical properties and manufacturability are no longer significant hurdles in developing bispecific clinical candidates. However, different formats do vary in the degrees of difficulty in Chemistry, Manufacturing and Controls (CMC) development, and the ones that fulfill developabil-
ity criteria no doubt would significantly lower development risk and shorten development timeline.

**Preclinical pharmacology and toxicology.** The preclinical pharmacology and toxicology studies are very critical for the development of bsAbs, as the results from these studies not only support the scientific rationale of the bsAbs but also provide valuable information for selecting the FIH dose. Though the scope of the bsAb preclinical studies may be similar to that for mAbs, the selection of the relevant species may be more challenging for bsAbs due to the additional target. The relevant species should be selected based on the following: (1) both targets should have similar expression profiles and biological functions as the targets in human, respectively, and (2) the bsAb should bind to both targets with similar properties as it binds to the human targets. In the case that Fc effector function is required, especially the ones with modified binding to FcγRs, the selected species should also be able to predict the Fc function in human. If such a species is available, the FIH dose may be selected based on the no-observed-adverse-effect level (NOAEL). If a relevant species is not available, in vitro pharmacology studies and in vivo pharmacology studies using surrogate bsAb or transgenic animals may be required to provide supporting information. The FIH dose may be selected by using the minimum anticipated biological effect level (MABEL) approach if no relevant species may be selected based on the following: (1) both targets should have similar expression profiles and biological functions as the targets in human, respectively, and (2) the bsAb should bind to both targets with similar properties as it binds to the human targets. In the case that Fc effector function is required, especially the ones with modified binding to FcγRs, the selected species should also be able to predict the Fc function in human. If such a species is available, the FIH dose may be selected based on the no-observed-adverse-effect level (NOAEL). If a relevant species is not available, in vitro pharmacology studies and in vivo pharmacology studies using surrogate bsAb or transgenic animals may be required to provide supporting information. The FIH dose may be selected by using the minimum anticipated biological effect level (MABEL) approach if no relevant toxicity species are available, especially for molecules with agonistic activities. Several case studies of the bsAb preclinical studies were reviewed by Prell et al. [173] and by Trivedi et al. [174] to illustrate the complexity and challenge during bsAb preclinical development.

**Clinical development.** Based on the draft guidance for bsAb development programs published by the FDA in April 2019, several factors should be considered during bsAb clinical development: (1) scientific rationale (e.g., MOA, therapeutic advantages over standard of care); (2) mode of action (e.g., bridge two target cells, simultaneous or sequential binding); (3) binding kinetics to each target; (4) special pharmacology studies (e.g., PK/PD assessment for active form of the bsAb, immunogenicity assessment for each domain of the bsAb); and (5) in certain cases, factorial design of clinical trials to inform risk/benefit ratio.

TGN1412, an anti-CD28 agonistic antibody case, alerted us that caution must be taken in regard to clinical development of bsAbs with novel MOAs, especially for agonistic molecules. Therefore, it is recommended that for bsAbs playing agonistic function, especially for unprecedented target pairs, the selection of the initial dose of the FIH trial should use MABEL approach. Additionally, agonistic bsAbs may have a bell-shaped dose-response that the therapeutic efficacy peaks at a dose that receptor occupancy is not saturated and then decreases along with the increased drug dose [175]. Therefore, an agonistic bsAb with a narrowed bell-shaped dose-response curve may be significantly difficult for researchers to select the optimal doses for different patients.

Comprehensive examinations for anti-drug antibodies may be required to evaluate the immunogenicity risk of different domains of the bsAbs, as some of the bsAbs are heavily engineered with potential immunogenic epitope introduced. Special attention needs to be taken on ADA against TRBAs and agonistic bsAbs using the tumor/tissue localization strategy, as the presence of ADA may break the TAA dependency of these bsAbs and lead to non-specific activation of immune cells and unpredictable severe adverse events. One should always follow FDA outlined and recommended adoption of a risk-based approach to evaluate and mitigate immune responses or adverse immunologically related responses associated with therapeutic protein products that affect their safety and efficacy during clinical development of a bsAb.

Furthermore, in some instances, combinational therapy provides the flexibility in adjusting the dosing regimen, which cannot be achieved by bsAbs. Although various bispecific formats can provide some degree of flexibility in adjusting affinity and valency of a binding specificity to suit different needs, once the format is determined, the ratio against two targets is fixed, and it cannot be adjusted based on the clinical results, which may pose clinical development challenge for a drug. Moreover, an optimal treatment may require sequential target intervention. For example, the concurrent treatment of anti-PD-1 with anti-OX40 treatment leads to substantial increase in serum cytokines and the expression of inhibitory receptors on T cells, as well as decreased T cell proliferation, thereby attenuating the antitumor efficacy of anti-OX40 treatment. However, delaying the PD-1 treatment can increase the antitumor activity of anti-OX40 treatment [176]. In another case, NK cells can be activated and upregulate 4-1BB expression by exposing to rituximab-coated CD20+ tumor cells or trastuzumab-coated Her2-overexpressing breast cancer cells. The anti-4-1BB treatment following the treatment of rituximab or trastuzumab can enhance the ADCC effect of NK cells to antibody-coated tumor cells [177, 178]. In such cases, the combinational therapy with mAbs offers the flexibility which cannot be accomplished by current bsAb strategies.

**Mechanistic or biology challenges**

The most fascinating applications of bsAbs are to enable novel biological function and therapeutic MOA otherwise impossible by using mAbs alone or in combination. However, the novel MOA may also impose unknown safety risk on bsAbs, which cannot be readily predicted or evaluated in preclinical studies, and possibly result in severe or even life-threatening adverse events during the clinical stage. Therefore, the uncertainty in function and safety of these bsAbs represents a major challenge for development of bsAb therapeutics.

When selecting the target pair, researchers should consider the spatial and temporal presence of both targets. Whether both targets are expressed at the same location at the same time? Whether their levels are within a reasonable range that can be effectively treated by a bsAb with fixed stoichiometry? Whether the two targets expressed on different cells or on the same cells? Whether the bsAb will mediate in-cis or in-trans engagement of the two targets? Will different engagement models result in different outcomes
in efficacy and safety? Those are all important questions one needs to think through when embarking on a bsAb project.

Bispecific antibodies engaging CD32B and FcεR were designed to employ the dominant negative role of CD32B and inhibit the activation of FcεR to alleviate IgE-mediated diseases. The bsAb 9202.1/5411 with IgG1 format was produced using Escherichia coli cell line and therefore had no Fc effector function due to lack of glycosylation. In vitro analysis showed that this bsAb can inhibit IgE-mediated activation of mast cells and basophils. As mentioned by the authors, several formats that were bivalent for FcεR might cross-link FcεR in the absence of CD32B, thereby activating rather than inhibiting FcεR [179]. One may speculate in the worst scenario in vivo, sometime may be inevitable, if such a molecule formed aggregates, it may function to activate rather than inhibit FcεR as one initially designed.

This becomes even more complicated for agonistic bsAbs to activate receptors. As a receptor is co-evolved with its cognate ligand, the signaling upon ligand-receptor engagement is evolved to be tightly controlled under physiological conditions. Due to the plasticity of receptors, polygamy widely exists for ligand-receptor interaction. When using antibody-based therapeutics to mimic the function of a ligand, the antibody may bind to the site on the receptor different from its cognate ligand binding site, which may elicit different signals. The deviation from the cognate activation may result in unexpected consequence, and their potential safety risk is unknown.

For example, as reported by Gu et al., a panel of biparatopic anti-Her2 antibodies in DVD-Ig format generated from the same parental mAbs only differed by VD orientations or linker length. Surprisingly, DVD-Ig molecules with one VD orientation showed agonistic effect and increased tumor cell proliferation, whereas molecules with the opposite VD orientation remained antagonistic. Further studies revealed that a particular VD orientation interrupted Her2/EGFR and Her2/Her3 interaction, resulting in increased Her2 homodimerization and activation [180]. Similarly, a biparatopic anti-CTLA-4 bsAb unexpectedly changed the signalosome assembly on the cytoplasmic domain of CTLA-4 and completely converted the inhibitory receptor into a stimulatory receptor [181].

The preclinical and clinical development path have largely paved for bsAbs with precedent mechanisms. However, the development of bsAbs with novel biological mechanisms still faces a few challenges and pitfalls. It may require more preclinical studies and early discussion with regulatory agencies for clinical development plans. We believe that, in the future, biology will be the key driver for design and selection of a bsAb and the key consideration for clinical development of bsAb drugs.

**PERSPECTIVE**

A growing number of recombinant bsAbs are now in clinical development. These bsAbs represent quite different formats. The number of the formats may reflect the diversity in desired features of therapeutic applications and may also reflect the different understanding of biology. For instance, for T cell-redirected cytotoxicity, a variety of formats, with differences in affinity, valency, domain geometry, Fc properties, and pharmacokinetic properties, have progressed into clinical development. It will be interesting to see clinical validation of various preclinical rationales behind the design of those molecules in the coming years.

With the advent of gene therapy, RNA therapy, cell therapy, and various other new therapies, we should always compare those different therapeutic options and pay close attention to those new therapeutic modalities that may have disruptive potentials, for instance, both chimeric antigen receptors T cell (CAR-T) therapy and TRBAs have demonstrated dramatic effects in patients with hematologic tumors. One TRBA and two CAR-T cell products have been approved by major regulatory agencies within the last 10 years for the treatment of hematological cancers, and an additional approximately 60 TRBAs and 300 CAR cell constructs are in clinical trials today. CAR-Ts are designed to activate T cells via intracellular T cell co-stimulatory signaling modules in tandem and to form a cytolytic synapse with target cells that is very different from the classical immune synapse both physically and mechanistically, whereas the TRBA-induced synapse is similar to the classic immune synapse by bringing T cells close proximity to tumor cells via a bispecific molecule. As published in 2018 ASH annual meeting, in patients relapsed refractory multiple myeloma (r/r MM), AMG-420 (BCMA × CD3, BiTE) showed 70% ORR and 40% CRR. Similarly, bb-2121 (BCMA CAR-T) and JCARH125 also demonstrated ~80% ORR and ~30% CRR. On the other hand, both TRBAs and CAR-T therapies showed similar adverse effect, which may be due to their MOA in redirecting T cell cytotoxicity to tumor cells. Blincyto and CAR-T therapies, Kymriah and Yescarta, are all targeting CD19+ tumor cells and proved for treatment of B cell lymphomas, and all of them have the block box warnings for CRS and neurological toxicities. From the manufacturing aspect, due to the characteristics of BiTE molecules, the manufacture of Blincyto still has quite a few challenges, but this has been solved by the next generation of TRBAs in the clinical development. For autologous CAR-T therapies, a complicated and time-consuming (3–4 weeks) manufacturing process is required for each patient. Additionally, as the CAR-T therapies are live cells, the regulatory requirements for CAR-T therapies are more complicated and stringent than regular biological therapeutics. Most CAR-T cells today are autologous, although significant strides are being made to develop off-the-shelf allogeneic CAR-based products. Therefore, in general comparing these two therapeutic platforms, TRBAs are the off-the-shelf products and may be more convenient and affordable to patients in the near future when more TRBAs are available, while CAR-T therapy may be tedious but may have advantage to mobilize the entire T cell machinery in a very different mechanism to fight cancer cells. Both platforms currently are facing the same moderate anticancer effects in solid tumor settings, probably due to inaccessibility of immune effector cells to solid tumors and complex immunosuppressive mechanisms at TME. The knowledge learned from clinical trials for either one will definitely help to improve the design of both.
therapies with additional immunomodulatory features to overcome the key challenges they are still facing. Nevertheless, bsAbs and msAbs open up tremendous opportunities to explore previously unexplored therapeutic options. We believe that the next decade will witness the clinical success of bsAbs or msAbs employing some novel MOAs in the applications in cancer and infectious, metabolic, ocular, and other diseases with significant unmet medical needs.

DECLARATIONS

Siwei Nie, Zhuozhi Wang, Jianqing Xu, and Jijie Gu are current employees of WuXi Biologics and may hold WuXi Biologics' stocks.

CONFLICT OF INTEREST STATEMENT

Siwei Nie, Zhuozhi Wang, Jianqing Xu and Jijie Gu are current employees of WuXi Biologics, and may hold WuXi Biologics' stocks.

ABBREVIATIONS:

- bsAb: bispecific antibody
- mAb: monoclonal antibody
- MPP: molecular product profile
- TPP: target product profile
- MOA: mechanism of action
- UMN: unmet medical needs
- TMDD: target-mediated drug disposition
- SDA: single-domain antibody
- Fv: variable fragment
- scFv: single-chain variable fragment
- Fab: antigen-binding fragment
- scFab: single-chain antigen-binding fragment
- VH: heavy chain variable domain
- VL: light chain variable domain
- CH1: heavy chain constant domain 1
- CH2: heavy chain constant domain 2
- CH3: heavy chain constant domain 3
- CH4: heavy chain constant domain 4
- Fe: fragment of crystallizable region
- FD: the heavy chain of a Fab, i.e. VH domain plus CH1 domain
- PK/PD: pharmacokinetic/pharmacodynamics
- TRBA: T cell-redirecting bispecific antibody
- TAA: tumor-associated antigen

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