https://doi.org/10.1146/annurev-chembioeng-100522-102155

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Annual Review of Chemical and Biomolecular Engineering

Mechanism-Driven Design of Multispecific Antibodies for Targeted Disease Treatment

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Keywords

antibody, protein engineering, immunotherapy, T cell, drug development, cancer

Abstract

Antibody-based therapeutics constitute a rapidly growing class of pharmaceutical compounds. However, monoclonal antibodies, which specifically engage only one target, often lack the mechanistic intricacy to treat complex diseases. To expand the utility of antibody therapies, significant efforts have been invested in designing multispecific antibodies, which engage multiple targets using a single molecule. These efforts have culminated in remarkable translational progress, including nine US Food and Drug Administration–approved multispecific antibodies, with countless others in various stages of preclinical or clinical development. In this review, we discuss several categories of multispecific antibodies that have achieved clinical approval or shown promise in earlier stages of development. We focus on the

molecular mechanisms used by multispecific antibodies and how these mechanisms inform their customized design and formulation. In particular, we discuss multispecific antibodies that target multiple disease markers, multiparatopic antibodies, and immune-interfacing antibodies. Overall, these innovative multispecific antibody designs are fueling exciting advances across the immunotherapeutic landscape.

1. INTRODUCTION

Over the past three decades, monoclonal antibodies have been employed widely as targeted therapeutics in the clinical setting, with applications in a broad spectrum of conditions, including cancers, infectious diseases, metabolic diseases, autoimmune diseases, and chronic inflammation (1). The hybridoma technology invented by Georges Köhler and César Milstein in 1975 enabled the production of pure monoclonal antibodies in large quantities, enabling scale-up manufacturing, which is needed for clinical adoption (2–4). The murine-derived anti-CD3 antibody denoted muromonab-CD3 (orthoclone OKT3) became the first therapeutic antibody to earn approval by the US Food and Drug Administration (FDA) in 1986, indicated for the prevention and treatment of acute allograft rejection after organ transplantation (5–8). However, clinical use of OKT3 was discontinued in 2010 due to severe side effects, such as life-threatening cytokine release syndrome (CRS), and the development of better-tolerated alternatives (9). Nonetheless, OKT3 introduced a revolutionary class of biologics to the clinic, and to date, more than 170 antibodies have been approved by at least one drug regulatory agency (10–12). However, the translation of monoclonal antibodies has been successful only for diseases with well-understood soluble or membrane protein targets that engage in known physiological functions (13, 14). To effectively treat other complex and poorly understood diseases with less accessible targets, there is immense interest in generating multispecific antibodies that contain two or more distinct binding domains (14, 15). Attempts to generate multispecific antibodies date all the way back to 1961, when Nisonoff & Rivers (16) recombined monovalent antibody fragments through chemical reoxidation.

Through their multifaceted activities, multispecific antibodies carry several functional advantages over conventional monoclonal antibodies, including (*a*) simultaneously regulating multiple signaling pathways implicated in disease pathogenesis; (*b*) increasing binding strength and selectivity; (*c*) reducing dose-dependent, off-target toxicity; (*d*) decreasing the likelihood of acquired resistance from monospecific therapeutics; (*e*) redirecting both innate and adaptive immune cells in close proximity to the target cells; and (*f*) exhibiting synergistic effects by recruiting multiple mechanisms of action (17–22). Antibodies enact a wide range of mechanisms, including neutralization, antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP), and multispecific antibodies allow the exciting opportunity to combine multiple complementary functions into a single molecule. Furthermore, compared to combining multiple individual monoclonal antibodies, multispecific antibodies can also reduce development and production costs (17, 23). As a result, efforts are underway to advance multispecific antibody development through discovery of novel targets, design of new formats, and improved manufacturing strategies.

More than 100 different formats of multispecific antibodies have been developed. These can be roughly divided into two categories: immunoglobulin G (IgG)-like (containing an Fc domain) and non-IgG-like (lacking an Fc domain) (1, 12, 13, 17, 23). IgG-like multispecific antibodies can be further grouped into two subcategories: asymmetric (comprising 2 distinct binding arms) and symmetric (homodimeric formats that introduce multiple specificities through

fusion of additional binding domains to an IgG-like molecule). The first multispecific antibody to gain clinical approval was Fresenius Biotech GmbH's catumaxomab (Removab®), which was approved by the European Medicines Agency in 2009 (24). Catumaxomab is an IgG-like bispecific antibody indicated for the treatment of malignant ascites caused by cancer (25, 26). It can bind tumor cells via an epithelial cell adhesion molecule (EpCAM)-specific arm and T cells via a CD3-specific arm (25–28). Catumaxomab was generated via quadroma by fusing an anti-EpCAM rat IgG2b hybridoma with an anti-CD3 mouse IgG2a hybridoma and was purified via a two-step chromatographic purification (27, 29). The fused Fc region adds additional functionality by allowing for engagement of immune effector cells via Fcγ receptors (FcγRs) (30, 31). Although catumaxomab was eventually withdrawn from the market (in 2017) due to immunogenicity and severe liver toxicity, this molecule ushered in a new wave of bispecific antibody therapeutics (1, 21, 32, 33). In 2015, Amgen's blinatumomab (Blincyto®), a bispecific T cell engager (BiTE) that targets CD3ε on T cells and CD19 on B cells, became the first FDA-approved bispecific antibody molecule, indicated for treatment of patients with relapsed or refractory B-precursor acute lymphoblastic leukemia (34–36). As of August 2023, there are 12 approved bispecific antibodies across the globe, including 9 FDA-approved molecules (10, 11, 37–39). In addition, more than 122 multispecific antibodies are currently undergoing clinical trials, including several trispecific antibodies.

In addition to their therapeutic applications, multispecific antibodies have also been used as clinical diagnostics and for medical imaging (34). The use of multispecific antibodies with enhanced target specificity, sensitivity, and selectivity can greatly augment the accuracy of clinical disease monitoring. For example, the bispecific antibody TF2 simultaneously recognizes histamine succinate glycine motif and carcinoembryonic antigen on cancer cells (40). When pre-targeted with radionuclide 68Ga-labeled bivalent histamine succinate glycine peptide, TF2 demonstrated improved sensitivity for tumor detection using immune-positron emission tomography in preclinical models (41). Moreover, a recent phase 1 trial demonstrated superior performance of this approach in clinical applications compared to monospecific antibody–based imaging (42).

Despite their vast potential as both diagnostics and therapeutics, multispecific antibodies face more complex challenges than monoclonal antibodies in terms of developability, manufacturing, and clinical translation. One of the most formidable hurdles in developing multispecific antibodies is enforcing proper heavy- and light-chain pairing to favor assembly of the desired antibody (21, 43–45). Over the past decades, numerous strategies have been developed to address this issue by promoting correct light-chain pairing (29, 46–54) or through heavy-chain heterodimerization strategies, such as knobs-into-holes (55–58). Other technologies include controlled Fab-arm exchange, which entails recombination after in vitro reduction (59–61), and the κλ body platform, which pairs a common heavy chain with either a kappa or lambda light chain (62). The requirement for simultaneous binding to multiple targets poses another developmental challenge for multispecific antibodies, because design parameters, such as format architecture and binding kinetics, may be interdependent (21). For example, the binding efficiency of one arm can be influenced by the binding valency or structural orientation of the second arm. Moreover, target distribution and availability of the binding epitope impact recognition and binding dynamics (63). As a result, laborious screening processes are required to identify optimal design features and appropriate topologies of binding arms that will achieve desired functional profiles (64, 65). The inherent complexity of multispecific antibodies also confers significant challenges in achieving favorable physical and chemical stability, solubility, viscosity, and pharmacokinetic properties (13, 18), while also avoiding immunogenicity. Other issues associated with the clinical developability

of multispecific antibodies include increased risk of immune effector cell–induced toxicity, such as CRS and neurotoxicity, as well as potential on-target off-tumor toxicity on healthy tissues with shared expression of the targeted antigens (18, 37).

The development of multispecific antibodies has advanced significantly in recent years due to rapid innovations in the areas of genetic engineering, protein engineering, and transgenic animal models. Several mechanisms that have been exploited for multispecific antibodies include (*a*) simultaneous blockade of multiple antigens and/or inhibition of associated signaling pathways; (*b*) manipulation of receptor clustering, internalization, target degradation, or activation; and (*c*) immune cell engagement and redirection (1, 20, 21, 43, 66) (**Figure 1**). In this review, we focus on mechanism-driven design of multispecific antibodies and discuss emerging strategies that are approaching clinical translation for targeted disease treatment.

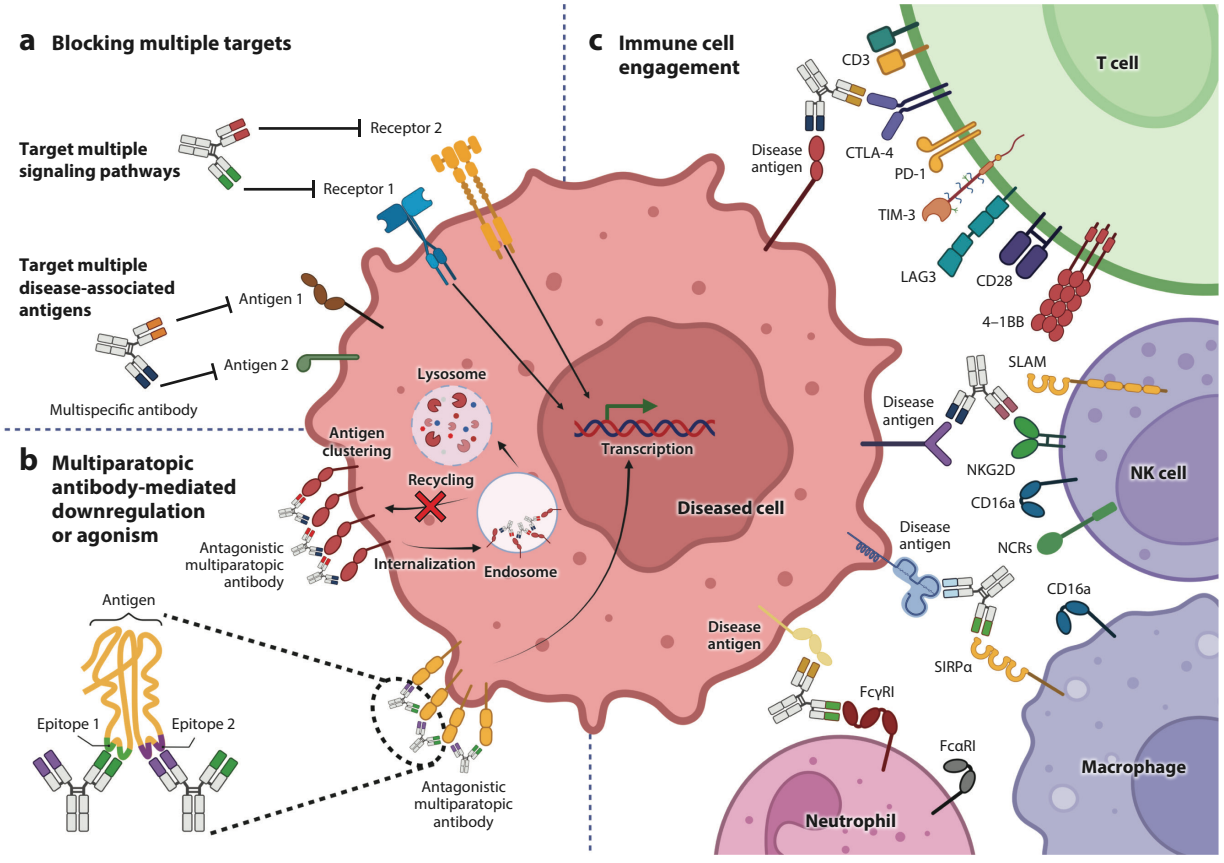


Figure 1

General representation of three mechanisms used by multispecific antibodies. (*a*) Blockade of multiple signaling pathways or disease-associated antigens to enhance therapeutic efficacy and target cell specificity. (*b*) Engagement of two or more nonoverlapping epitopes on the same target cell surface protein to facilitate downregulation and/or signal agonism. (*c*) Redirection of adaptive or innate immune cells to recruit clearance mechanisms to the site of disease. Abbreviations: CTLA-4, cytotoxic T lymphocyte antigen-4; FcαRI, Fc alpha receptor 1; FcγRI, Fc-γ receptor 1; LAG3, lymphocyte activation gene 3 protein; NCR, natural cytotoxicity receptor; NK, natural killer; NKG2D, natural killer group 2D; PD-1, programmed cell death protein 1; SIRPα, signal-regulatory protein α; SLAM, signaling lymphocytic activation molecule; TIM-3, T cell immunoglobulin and mucin-domain containing-3. Figure adapted from images created with BioRender.com.

2. USING MULTISPECIFIC ANTIBODIES TO TARGET MULTIPLE DISEASE MARKERS WITH A SINGLE MOLECULE

2.1. Targeting Receptor Signaling Pathways in Cancer

One important application of multispecific antibodies is concurrent targeting of multiple signaling pathways. This approach can be beneficial over traditional monospecific antibody therapies in three ways: (*a*) Targeting redundant signaling pathways can more potently abrogate signal transduction; (*b*) inhibiting synergistic signaling pathways can prevent or overcome drug resistance; and (*c*) targeting upregulated receptors can facilitate engagement with co-expressed, less-abundant receptors.

In the context of cancer, bispecific antibodies have shown promise in overcoming acquired resistance to antitumor drugs. For example, in non-small cell lung cancer (NSCLC), activating mutations in the receptor tyrosine kinase epidermal growth factor receptor (EGFR) cause upregulated tyrosine kinase activity (67). First-line treatments for these mutated cancers include gefitinib and erlotinib, both of which are small molecules that act as tyrosine kinase inhibitors. However, drug resistance limits response to these drugs to a mean duration of <1 year (68, 69). Increased expression of cMet pathway components fulfills the role of a tyrosine kinase, bypassing tyrosine kinase inhibitor–mediated blockade of EGFR signaling (70–72). To overcome this challenge, Janssen Biotech, Inc. (73) developed a bispecific antibody, amivantamab (or Rybrevant®), that targets both EGFR and cMet. Amivantamab is a fully humanized human IgG1 antibody that combines two monospecific antibodies with complementary Fc domain mutations using controlled Fab-arm exchange, wherein the antibodies are reassembled following in vitro reduction (60). Amivantamab was granted accelerated approval from the FDA and was also approved for use in the European Union in 2021. The bispecific format was designed to have multiple mechanisms of action: (*a*) concurrent inhibition of both receptors, (*b*) induced receptor degradation, and (*c*) destruction of tumor cells via Fc effector function (73). In this case, the bispecific format was necessary to block both redundant pathways, as well as to specifically target tumor cells that upregulated both of these receptors on their surface. Of particular note, this bispecific antibody was prepared under such conditions that it lacked fucosylation, resulting in tighter binding of FcγRIIIa and thereby enhancing immune effector function (74).

Another class of malignancies wherein first-line treatments fail is human epidermal growth factor 2 (HER2)-driven cancers, including breast cancers, ovarian cancers, squamous cell carcinomas of the head and neck, and NSCLC (see references in 75). In HER2-driven cancers, targeted therapy relies on kinase inhibition of the phosphatidylinositide-3 kinase (PI3K)/Akt pathway. However, HER3 hyperactivation, through heterodimerization with HER2, can also activate the PI3K/Akt pathway, even when HER2 is inhibited. To overcome this resistance, Merus (75) developed a bispecific antibody denoted zenocutuzumab (MCLA-128), targeting both HER2 and HER3. Zenocutuzumab is a humanized full-length IgG1 antibody comprising two different variable domains, and it is currently undergoing testing in phase 2 clinical trials for treatment of patients with solid tumors harboring NRG1 gene fusions (76). In this case, the bispecific format is necessary to block dimerization of two surface receptors, which is not easily achieved using a monospecific format.

In addition to orchestrating cancer cell killing, multispecific antibodies can be used to target cancer cell metastasis. Concentrations of the cytokine interleukin-6 (IL-6) and the chemokine IL-8 were shown to be elevated in the serum of patients with solid tumors whose disease metastasized to the lung and liver (77–79). These soluble factors signal through their cognate receptors, IL-6 receptor α (IL-6Rα) and gp130 for IL-6, and IL-8RA or IL-8RB for IL-8. Signaling through the IL-6 and IL-8 pathways has been linked to metastasis in many cancer cell types, including

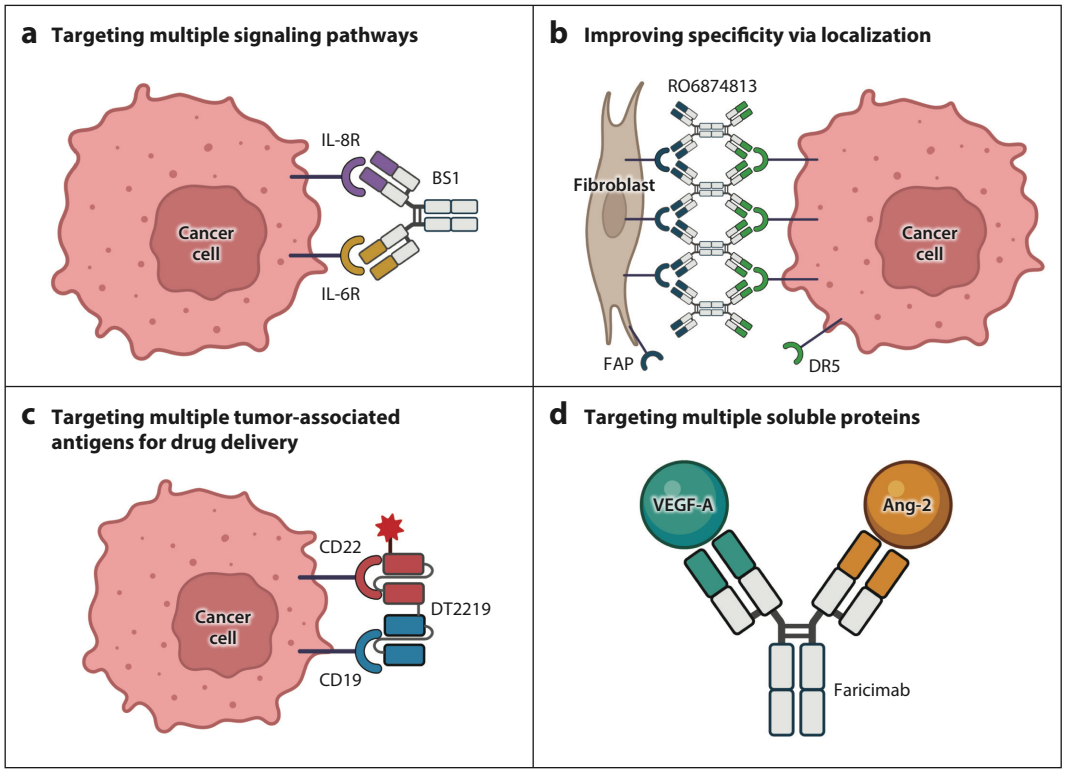


Figure 2

Examples of multispecific antibodies targeting multiple disease markers. (*a*) The antimetastatic bispecific antibody BS1, which targets IL-6Rα and IL-8RB (denoted IL-6R and IL-8R, respectively). (*b*) The bispecific antibody RO6874813, which induces DR5 clustering in the tumor microenvironment via interaction with FAP on tumor-associated fibroblasts. (*c*) The bispecific immunotoxin DT2219, which delivers diphtheria toxin to cancerous B cells. (*d*) The bispecific antibody faricimab, which blocks the activity of VEGF-A and Ang-2 by simultaneously neutralizing these soluble proteins. Abbreviations: Ang-2, angiopoietin-2; DR5, death receptor 5; FAP, fibroblast activation protein; IL-6Rα, interleukin-6 receptor alpha; IL-8RB, interleukin-8 receptor beta; VEGF-A, vascular endothelial growth factor A. Figure adapted from images created with BioRender.com.

triple-negative breast cancer (80). To concurrently inhibit these two synergistic signaling pathways, two bispecific antibodies (BS1 and BS2) were developed that bind the IL-6 and IL-8 receptors concurrently to antagonize them through preventing their activation by the respective ligands (81) (**Figure 2*a***). This format includes the variable domains of the neutralizing commercial anti-IL-6Rα antibody tocilizumab (Actemra®; Roche) and the neutralizing anti-IL-8RB antibody 10H2 (82). Preclinical in vitro migration and in vivo mouse studies demonstrated that metastasis was potently reduced by the bispecific antibody, more significantly than was observed with combination therapy comprising tocilizumab and the small-molecule IL-8R inhibitor reparixin (81). Notably, IL-8RB was significantly upregulated in triple-negative breast cancer tissue samples, whereas IL-6Rα was upregulated only marginally. Thus, the bispecific format enabled targeting of a low-expression receptor that was inaccessible to a monospecific antibody. Additionally, the bispecific format outperformed combination therapy with a monospecific antibody and a small molecule, both enhancing therapeutic efficacy and circumventing the clinical barriers of dose optimization and off-target toxicity of small molecules.

2.2. Combinatorial Targeting of Tumor-Associated Antigens

Another application of multispecific antibodies is leveraging tumor-associated antigens for specific targeting of tumor cells. Often, tumor cells upregulate certain surface markers that do not necessarily have exploitable pathogenic functions. Targeting these surface markers localizes a bispecific antibody to tumor cells, facilitating engagement with another functional receptor that may be expressed at lower densities.

A promising class of anticancer targets are apoptotic pathway components, including those of the extrinsic apoptotic pathway. One of the receptors in this pathway is death receptor 5 (DR5), which upon activation oligomerizes and promotes formation of the death-inducing signaling complex (83, 84). Many antibodies have been developed to target this pathway but have demonstrated limited clinical efficacy (85–88). One of the main reasons these therapies fail is due to the need for crosslinking to achieve oligomerization of DR5, which could be addressed through multivalent presentation of antibody variable domains and improved localization to the tumor microenvironment. Fibroblast activation protein (FAP) is a membrane protein expressed in reactive stromal fibroblasts of many epithelial cancers, including breast, colorectal, and lung cancers (89–91), and serves as a robust localization marker to cancer cells. To take advantage of the tumor-specific expression of FAP, the bispecific antibody RO6874813 (RG7386, developed by Hoffman-La Roche) was designed to target both FAP and DR5 (92) (**Figure 2*b***). The format of RO6874813 is a tetravalent fusion of an anti-FAP antibody and an anti-DR5 antibody. Thus, RO6874813 is able to utilize FAP for localization to the tumor microenvironment and oligomerize DR5 through its bivalency for this second target to induce apoptosis of cancer cells. This molecule is currently undergoing a phase 1 clinical trial for patients with locally advanced and/or metastatic solid tumors.

Bispecific antibody approaches can also be used to localize therapeutic cargo to cancer cells through targeting multiple tumor-associated antigens. This is achieved through the formation of antibody/toxin fusion proteins known as immunotoxins (93). Antibodies have been fused to a variety of cell-killing toxins, such as ribosome-inactivating proteins (RIPs), pore-forming toxins (PFTs), and microtubule-disrupting proteins (MDPs) (94). A novel bispecific antibody-based immunotoxin, DT2219 (developed by the Masonic Cancer Center, University of Minnesota), is composed of tandem antibody single-chain variable fragments (scFvs) targeting CD19 and CD22 fused to the catalytic and translocation-enhancing domains of diphtheria toxin (DT) (95) (**Figure 2*c***). This molecule showed potent killing of B-cell lymphoma and leukemia cells in vitro, robust cell killing in an in vivo flank tumor model, and improved survival in a mouse xenograft model of human leukemia (95). Building on these promising findings, a phase 1 clinical study of DT2219 was initiated in patients with relapsed/refractory B-cell lymphoma or leukemia. The results were promising, demonstrating the safety of DT2219 and defining the optimal dosing strategy (96). Following the successful phase 1 trial, a phase 1/2 clinical trial was initiated and showed promising preliminary results (97). In this case, the bispecific format achieved enhanced specificity for cancerous B cells expressing both CD19 and CD22, thus improving on-target cell killing via DT in comparison to monospecific immunotoxins.

2.3. Use of Bispecific Antibodies in Non-Cancer Disease Applications

In addition to cancer, multispecific antibodies have been developed to treat a range of other diseases, including two FDA-approved molecules. The first bispecific antibody approval in the United States in a non-oncology application was for hemophilia A, an X-chromosomal recessive genetic disorder of bleeding caused by mutation of an F8 gene encoding for clotting factor VIII (FVIII) (98). Due to deficiencies in FVIII, FIXa cannot bind to and activate FX, disrupting blood clotting. To address the lack of FVIII, a humanized bispecific antibody, denoted emicizumab (Hemlibra®;

Roche), was developed that binds both FIXa and FX (99). Emicizumab functions as a substitute for the cofactor activity of FVIII, restoring the clotting pathway by enforcing interaction between FIXa and FX. Emicizumab was approved by the FDA in 2017 for prophylaxis in adult and pediatric hemophilia A patients with or without FVIII inhibitors (100, 101). Prior to emicizumab approval, hemophilia A was typically treated via replacement therapy with recombinant FVIII. However, a key challenge associated with this treatment is the development of autoantibodies against FVIII, which occurs in up to 25% of treated patients (100). Thus, emicizumab bypasses FVIII autoantibody risk and shows minimal immunogenicity.

The second bispecific antibody approval in the United States in a non-oncology application was for wet age-related macular degeneration (w-AMD) and diabetic macular edema (DME), two highly prevalent and devastating ocular diseases. In these conditions, the current first-line treatment involves intravitreal drugs targeting vascular endothelial growth factor A (VEGF-A), which is believed to play a critical role in disease pathogenesis (102). However, these treatments are expensive and require frequent and invasive injections, and treatment efficacy is variable (103–105). As a result, alternative treatment strategies were investigated, such as those involving the angiopoietin (Ang) tyrosine kinase endothelial receptors (Tie) pathway (106). This pathway is involved in regulation of vasculature, and binding of Ang-2 to its cognate receptor Tie-2 leads to vascular leakage (107). Roche developed the bispecific antibody faricimab (Vabysmo®), which targets both VEGF-A and Ang-2 (108) (**Figure 2*d***). Faricimab was approved by the FDA in 2022 and demonstrates another case in which simultaneous binding of two soluble proteins leads to therapeutic efficacy. Of particular note, this strategy involves inhibition through binding of soluble ligands and not their cognate membrane-bound signaling receptors.

3. MULTIPARATOPIC ANTIBODIES: NEXT-GENERATION APPROACHES TO MECHANISM-DRIVEN THERAPEUTICS

A major advantage of antibody therapeutics, and particularly multispecific antibody drugs, is their recruitment of multiple complementary mechanisms (109). Another mechanism for antibody therapeutics that has received growing attention in conditions such as cancer and infectious diseases is manipulation of protein trafficking to induce target downregulation. In particular, whereas a monospecific antibody can dimerize a target antigen due to its specificity for a single epitope (**Figure 3*a***), combinations of two monospecific antibodies targeting two noncompetitive epitopes on the same target protein can induce clustering, leading to synergistic target downregulation and enhanced degradation that antagonize receptor activity (110–113). Friedman et al. (111) first demonstrated this phenomenon in the EGFR system by showing that the combination of two noncompetitive anti-EGFR antibodies induced significant receptor internalization and degradation without inducing receptor activation, whereas treatment with a single monoclonal antibody did not lead to changes in receptor trafficking. Building on this seminal work, Spangler et al. (114) defined the requirements for antibody-induced downregulation in the EGFR system. Specifically, the investigators showed that combining two noncompetitive antibodies targeting EGFR domain 3 resulted in receptor clustering and downregulated surface EGFR levels by up to 80% with a downregulation halftime of 0.5–5 h in both normal and transformed human cell lines. This downregulation mechanism resulted from abrogation of receptor recycling, and importantly, internalization was achieved in the absence of receptor activation. Inspired by antibody cocktail-induced EGFR downregulation, Spangler et al. (115) designed a panel of constructs that combined the anti-EGFR antibody cetuximab (Erbitux®) with EGFR-targeted fibronectin type III domain moieties, formulating biparatopic, triparatopic, and even tetraparatopic antibody/fibronectin type III domain fusion proteins. Among the engineered multiparatopic formats,

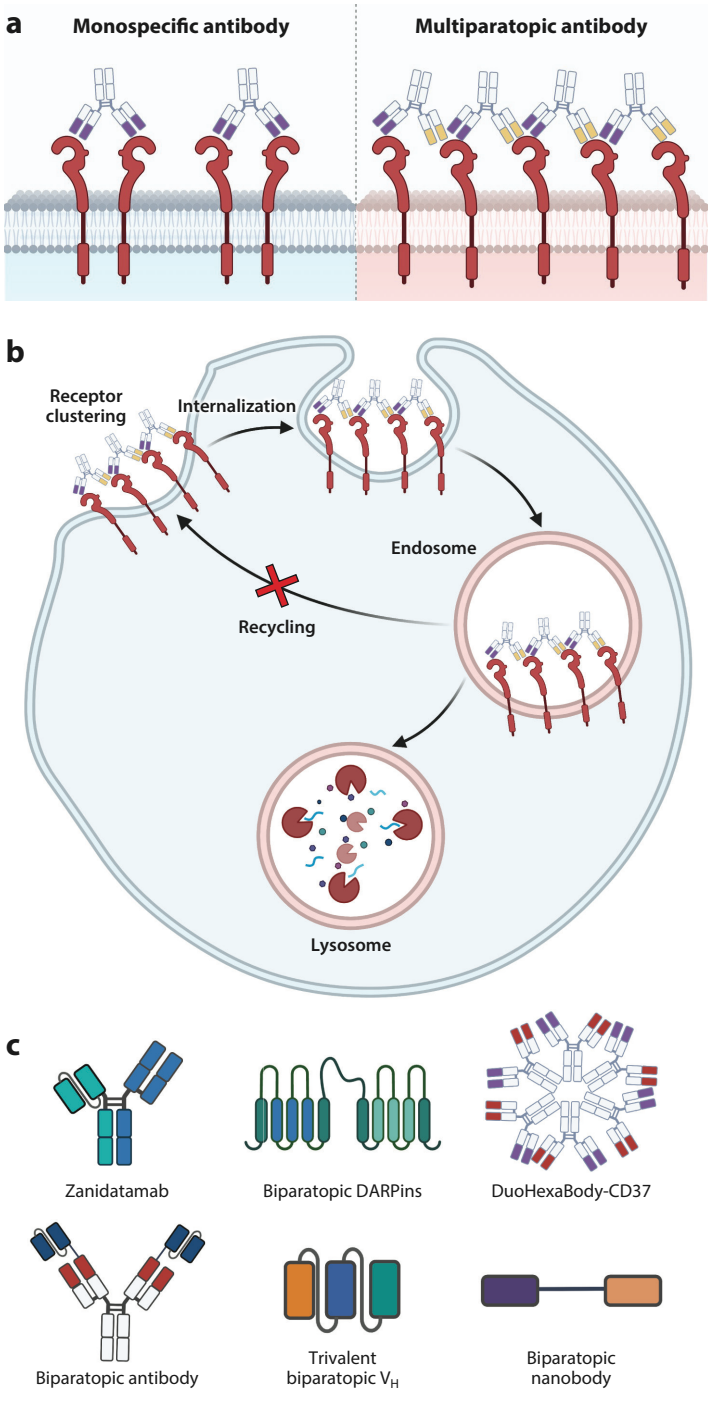


Figure 3

Antibodies mediate crosslinking of transmembrane proteins. (*a*) Monospecific antibodies bind to a single epitope on the target cell surface antigen and are thus limited to formation of dimers. (*b*) In contrast, multiparatopic antibodies bind to two or more nonoverlapping epitopes on the same target cell surface antigen, inducing clustering and crosslinking, which modulates antigen trafficking, enhancing internalization and inhibiting recycling to increase lysosomal degradation. (*c*) Schematic illustration of various multiparatopic antibody formats. Abbreviations: DARPin, designed ankyrin repeat protein; V_H, heavy-chain variable domain. Figure adapted from images created with BioRender.com.

triparatopic antibody fusion proteins demonstrated optimal efficacy, inducing rapid EGFR clustering and internalization without activating the receptor, and thus ablating downstream signaling. Of note, combined EGFR signaling inhibition and downregulation led to enhanced antitumor activity in mice, including in models that were resistant to treatment with the parent cetuximab antibody due to activating mutations in downstream signaling proteins.

Proof-of-concept validation for EGFR downregulation inspired later studies targeting other oncogenic growth factor receptors, such as HER2. In the case of the anti-HER2 antibody trastuzumab, which is clinically approved for the treatment of $HER2^+$ breast cancer, Gennari and colleagues (116) administered the drug to 11 patients with $HER2^+$ breast tumors at a standard dose for 4 weeks and found no significant downregulation of HER2. Additionally, Austin et al. (117) observed through in vitro studies using SKBr3 cells that trastuzumab did not downregulate HER2 due to efficient receptor recycling after endocytosis. The combination of trastuzumab with another noncompetitive anti-HER2 drug, pertuzumab, demonstrated improved antitumor efficacy in patients with $HER2^+$ metastatic breast cancer, as well as in patients with early breast cancer (118, 119). Mechanistic studies revealed that dual targeting of HER2 by the combination of trastuzumab and pertuzumab induced strong HER2 downregulation, whereas in vitro and in vivo treatments with either monospecific antibody alone did not significantly downregulate HER2 (120–122). Because translation of combination antibody therapies is logistically challenging due to the need for dosing ratio optimization and additional formulation considerations, there has been a push toward the development of multiparatopic antibodies to efficiently downregulate surface receptors in a unimolecular format. Multiparatopic antibodies represent a class of multispecific antibodies that engage two or more nonoverlapping epitopes on the same target protein. Whereas a monospecific antibody can crosslink only two receptors, a multiparatopic antibody can form theoretically infinite chains of crosslinked receptors (**Figure 3*a***), which can modulate molecular trafficking (**Figure 3*b***). In the case of HER2, biparatopic antibodies led to enhanced internalization and increased degradation, likely due to receptor crosslinking (114, 123, 124). Zanidatamab (ZW25 from Zymeworks) is a humanized IgG1-like biparatopic antibody, constructed by combining an scFv targeting extracellular domain 4 of HER2 and a Fab targeting extracellular domain 2 (the dimerization domain) of HER2 (124) (**Figure 3*c***). This construct parallels the specificity of combination treatment with trastuzumab and pertuzumab, which target extracellular domains 4 and 2, respectively. Compared to treatment with either monospecific antibody alone or the combination of trastuzumab and pertuzumab, zanidatamab strongly neutralized HER2 activity, and zanidatamab led to the formation of large HER2 clusters on the cell surface that enhanced HER2 internalization and downregulation. Zanidatamab was found to inhibit both cell signaling and tumor growth, while enhancing ADCC and ADCP, showing superior antitumor activity compared to trastuzumab/pertuzumab combination treatment in a $HER2^+$ patient-derived xenograft model of gastric cancer (124). In a phase 1 trial, zanidatamab was evaluated for safety, tolerability, and antitumor activity in patients with HER2-expressing tumors (125). The biparatopic antibody was found to be well tolerated and demonstrated promising antitumor activity in patients with advanced HER2-overexpresing tumors. A phase 2 trial investigated zanidatamab combined with chemotherapy in patients with HER2-expressing metastatic gastroesophageal adenocarcinoma. The results showed significant and durable responses with 84% overall survival at 18 months (126). Currently, zanidatamab is undergoing a phase 3 trial (NCT05152147) in combination with chemotherapy plus or minus tislelizumab [an anti-programmed cell death protein 1 (PD-1) antibody from Novartis] as a first-line treatment for HER2-expressing metastatic gastroesophageal adenocarcinoma.

Other HER2-targeted efforts predating zanidatamab involved the design of multiparatopic binders based on non-immunoglobulin-based binding scaffolds, in particular, designed ankyrin

repeat proteins (DARPins). Biparatopic DARPins (consisting of two binding units connected by a short flexible linker) (**Figure 3*c***) that recognize extracellular domains 1 and 4 of HER2 were shown to induce formation of inactive HER2 dimers and enforce oligomerization through crosslinking, preventing kinase domain autophosphorylation and consequent receptor activation. Accordingly, biparatopic DARPins potently induced apoptosis in HER2-addicted breast cancer cell lines (127). Encouraged by the therapeutic potential of the biparatopic DARPin constructs, Molecular Partners has developed a drug called MP0274, which is composed of two DARPin modules targeting nonoverlapping epitopes on HER2 and two additional DARPin modules targeting albumin to increase serum stability (128). Based on promising preclinical cancer models, MP0274 is being tested in a phase 1 clinical study of patients with $HER2^+$ solid tumors (129).

Another cancer antigen, CD37, has gained renewed interest as a promising therapeutic target for B cell malignancies, due to its selective expression on mature B cells with limited or no expression on other hematopoietic cell types, such as T cells, natural killer (NK) cells, granulocytes, monocytes, and dendritic cells (130–136). Oostindie et al. (137) developed a novel anti-CD37 biparatopic antibody called DuoHexaBody-CD37 (**Figure 3*c***) that targets two nonoverlapping epitopes on CD37 and contains an E430G hexamerization-enhancing mutation in the Fc domain to boost CDC. DuoHexaBody-CD37 demonstrated superior CDC activity both in vitro and in vivo compared to administration of anti-CD37 antibody variants alone or in combination (137). In addition to HER2 and CD37, the multiparatopic antibody concept has also been successfully extended to increase endocytosis and lysosomal degradation of various surface proteins, including mesenchymal epithelial transition (MET) factor, major histocompatibility complex (MHC) class I molecules, transferrin receptor, and the glycosyl-phosphatidylinositol (GPI)-linked enzyme CD73 (115, 138–144).

3.1. Multiparatopic Antibody–Drug Conjugates

Because multiparatopic strategies enhance antibody internalization through antibody-mediated clustering and crosslinking, these approaches can be particularly useful for the delivery of therapeutic payloads. Antibody–drug conjugates (ADCs) represent a growing class of molecular therapies that link an antibody to a potent cytotoxic drug. To date, the FDA has approved a total of 13 ADCs, and >1,000 molecules are in preclinical or clinical development (145), all for treatment of cancer. ADCs combine the advantages of specific target binding (conferred by the antibody) and potent cell killing (conferred by the cytotoxic drug) for robust and durable elimination of tumor cells (146). A crucial step in ADC-mediated cancer cell killing is internalization of the molecule into the target cell. Whereas some target proteins are endocytosed readily, other proteins persist on the surface, limiting their potential as effective ADC targets. Due to their capacity to induce antibody-mediated cell-surface protein internalization, multiparatopic antibodies have been recognized as potential candidates in ADC development. Li et al. (66) exploited this strategy by conjugating a tubulysin-based microtubule inhibitor to a biparatopic antibody against HER2 that induces receptor clustering, internalization, and degradation. The biparatopic antibody attaches the trastuzumab scFv to the N terminus of the heavy chain of the noncompetitive fully human antibody 39S IgG (**Figure 3*c***). The resulting biparatopic ADC induced rapid and robust HER2 receptor internalization, and the internalized complexes were found to be colocalized with lysosomes (66). Moreover, the biparatopic ADC demonstrated superior antitumor activity compared to the clinically approved trastuzumab-based ADC ado-trastuzumab emtansine (Roche's Kadcyla®) in preclinical tumor models (124). Based on these encouraging findings, the biparatopic ADC entered a phase 1 clinical trial (NCT02576548) for treatment of advanced breast and gastric cancers under the name MEDI4276, developed by MedImmune LLC. Although clinical activity

of MEDI4276 was observed in this trial, toxicity and neuropathy were observed in several patients (147), leading to discontinuation of research on this drug (148). Another recently developed anti-HER2 biparatopic ADC is zanidatamab zovodotin (ZW49), developed by Zymeworks, which conjugates the biparatopic antibody zanidatamab to the microtubule inhibitor monomethyl auristatin E (MMAE) via a protease-cleavable linker (149). Preclinical results showed promising efficacy in breast cancer cell lines expressing low and high levels of HER2, as well as in patient-derived xenograft models in mice (150). Based on these findings, ZW49 is now under investigation in a phase 1 clinical trial in patients with locally advanced or metastatic HER2-expressing cancers.

3.2. Multiparatopic Antibodies for Treatment of Infectious Diseases

In addition to targeting tumor-associated antigens, multiparatopic antibodies have been functionally extended to target infectious diseases, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-19. Bracken et al. (151) constructed a synthetic variable heavy chain (V_H) phage-displayed library based on the V_H domain of the anti-HER2 antibody trastuzumab to isolate binders against the SARS-CoV-2 spike protein receptor-binding domain (RBD) that interface with angiotensin-converting enzyme 2 (ACE2), which is expressed on host cells and serves as the portal for viral entry. The investigators identified V_H binders against two nonoverlapping epitopes on the spike protein RBD and linked them to generate biparatopic binders. Compared to single V_H domains, these biparatopic constructs exhibited increased affinity for RBD (up to 600-fold improvement) and augmented neutralization potency on pseudotyped SARS-CoV-2 virus (up to 1,400-fold). The most potent binder, a trivalent biparatopic V_H (**Figure 3*c***), neutralized SARS-CoV-2 with a half-maximal inhibitory concentration of 4.0 nM (151). In another study, Wagner et al. (152) isolated high-affinity noncompetitive neutralizing nanobodies specific for the SARS-CoV-2 spike protein RBD. The antibodies were assembled to construct two biparatopic tandem nanobody fusion proteins (denoted NM1267 and NM1268; **Figure 3*c***) targeting a conserved epitope outside and two distinct epitopes within the ACE2 interface. In vitro studies demonstrated that NM1267 and NM1268 were thermally stable and bound all circulating variants of concern. Additionally, in vivo studies revealed that mice treated with biparatopic nanobody fusion proteins showed significantly reduced disease progression and increased survival rates (152).

3.3. Agonistic Multiparatopic Antibodies

Besides target downregulation and neutralization of infectious diseases, biparatopic antibodies also function as agonist antibodies targeting immune checkpoint proteins, such as those in the tumor necrosis factor receptor (TNFR) superfamily (153). These agonist antibodies represent an important class of emerging immunotherapeutic agents that have demonstrated great potential in cancer treatment. The TNFR superfamily has been recognized as key players in cell differentiation and proliferation (154), and activation of these receptors on immune cells promotes antitumor effects (153, 154). TNFR clustering is a critical mechanism that triggers receptor activation; however, monospecific antibodies have demonstrated limited ability to mediate receptor clustering on the cell surface (155). In a recent study, Yang et al. (156) engineered biparatopic tetravalent antibodies targeting the OX40 receptor to elucidate their effects on $CD4^+$ T cell proliferation. These biparatopic tetravalent antibodies led to >3-fold improvement in $CD4^+$ T cell proliferation compared to their monospecific counterparts in the absence of CD28 costimulation and without FcγR-mediated crosslinking. In another notable example of antibody-induced agonism through targeting multiple epitopes, treatment with the combination of two anti-DR5 antibodies that target nonoverlapping epitopes (each containing hexamerization-enhancing Fc domain mutations)

led to efficient DR5 signaling and caspase-mediated cell death in the absence of FcγR-mediated crosslinking (157). Based on promising preclinical results across multiple cancer models, this therapy (known as HexaBody-DR5/DR5, GEN1029, developed by Genmab) is currently being tested clinically in patients with solid malignant tumors (NCT03576131). Collectively, multiparatopic antibodies represent an innovative class of molecules that recruit novel mechanisms related to protein trafficking, which enables applications including signal downregulation, ADC enhancement, neutralization of infectious diseases, and immune agonism.

4. IMMUNE-INTERFACING MULTISPECIFIC ANTIBODIES

A major category of multispecific antibodies encompasses molecules intended to enforce interactions between the immune system and disease targets. In fact, the first FDA-approved multispecific antibody, blinatumomab, falls into this category, and countless others have followed suit. Here, we review developments in the design of immune-recruiting multispecific antibodies, including T cell engagers, immune checkpoint inhibitors, co-stimulatory agonists, and innate immune cell engagers.

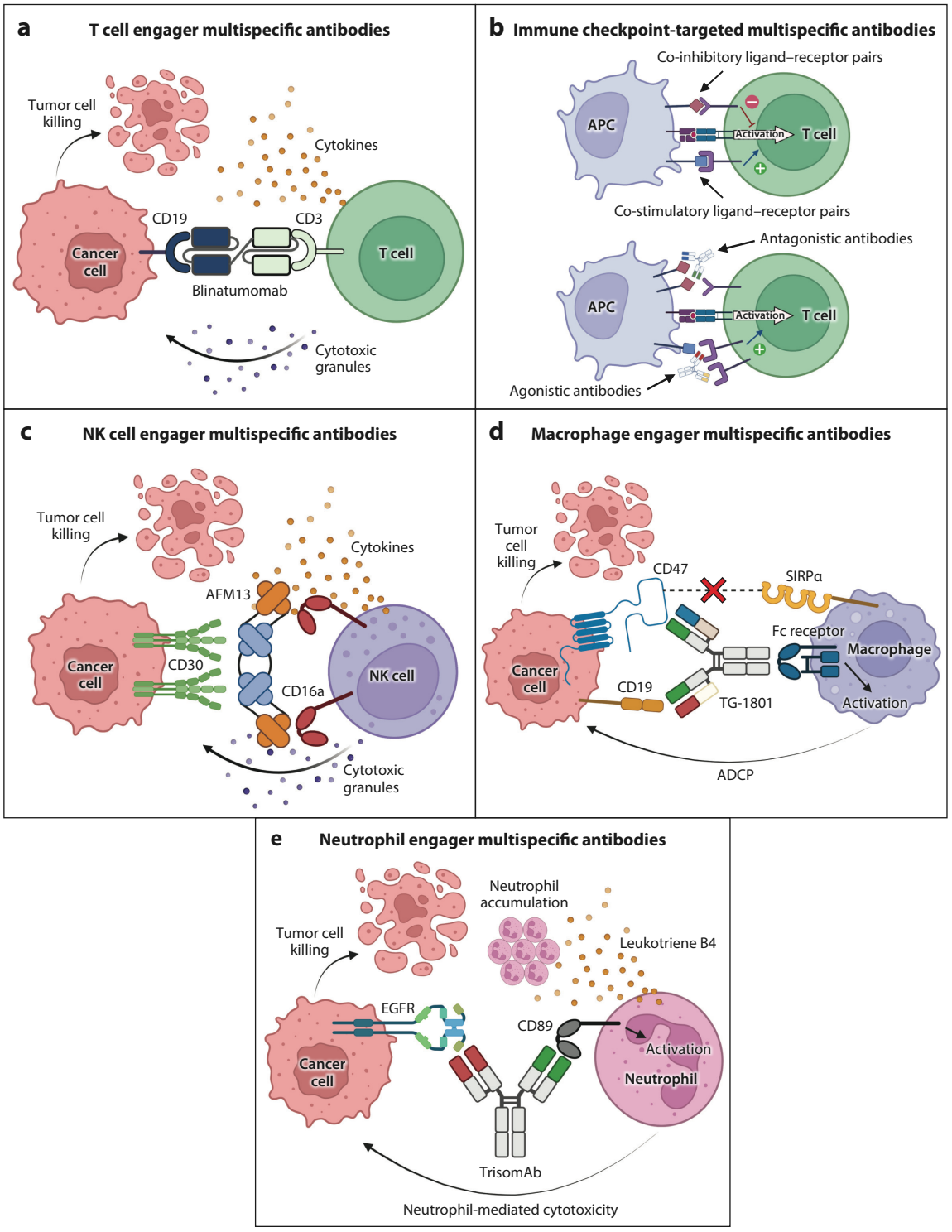
4.1. Using Anti-CD3 Multispecific Antibodies as T Cell Engagers

One important subset of immune-interfacing multispecific antibodies is T cell engagers. In this format, one antibody site binds CD3, a marker for T cells (both $CD4^+$ and $CD8^+$), whereas other site(s) are used to localize T cells to the target(s) of interest. CD3 is a co-stimulatory molecule, and thus crosslinking CD3 with a bivalent antibody supports T cell activation. Interestingly, use of a multispecific antibody to monovalently engage CD3 on T cells and simultaneously bind to an antigen on a target cell can also lead to T cell activation. A frequent application of this approach is that of targeting T cells to cancer cells to generate tumor-specific immune responses. As discussed in the introduction, the first T cell engager approved for clinical use was catumaxomab, an anti-CD3 and anti-EpCAM bispecific trifunctional antibody (158). Although this molecule was later withdrawn from the market, catumaxomab established an exciting new class of anti-CD3 multispecific antibodies, which has now grown to six clinically approved molecules in the United States.

One notable class of anti-CD3 bispecific antibodies is BiTEs, fusion proteins consisting of two tandem scFvs that are tethered together, with no Fc domain. Blinatumomab, which targets CD3 and CD19, remains the only clinically approved BiTE to date (159) (**Figure 4*a***). Because CD19 is a surface marker expressed on precursor B cells that is implicated in self-renewal of cancerous cells in hematologic malignancies, blinatumomab is FDA approved for the treatment of human B-precursor acute lymphocytic leukemia (160, 161).

Although BiTEs have shown promising efficacy, their activity is accompanied by safety concerns, most prominently CRS, which results in systemic and pathogenic immune activation. Moreover, the low serum half-life of BiTEs necessitates elevated and continuous dosing levels, leading to increased likelihood of adverse immune effects. In the case of blinatumomab, the drug is administered as a continuous infusion for four to eight weeks (162), and CRS has been observed (163). In addition, it was determined that the dose-limiting toxicity for blinatumomab was neurotoxicity (164). To mitigate these effects, different step-up dosing regimens have been used, which has decreased incidence of these symptoms (165).

Despite safety concerns associated with BiTEs, their clinical performance has validated bispecific T cell engagement as an effective anticancer strategy, motivating the development of new antibodies that use the same mechanism as BiTEs in a less toxic format. One example is mosunetuzumab (Roche's Lunsumio®), a humanized full-length anti-CD3/CD20 bispecific antibody,



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Examples of immune-interfacing multispecific antibodies. (*a*) A BiTE antibody, blinatumomab, which colocalizes T cells and cancerous B cells and induces tumor cell killing. (*b*) Immune checkpoint protein-targeted antibodies that either block inhibitory ligand–receptor pairs or agonize stimulatory ligand–receptor pairs to empower APC-mediated activation of T cells. (*c*) An NK cell–recruiting bispecific antibody, AFM13, which binds CD16a on NK cells to facilitate their destruction of CD30-expressing malignant cells. (*d*) A bispecific κλ body, TG-1801, which recruits macrophages through Fc receptor interactions and simultaneously engages CD19 and CD47 on tumor cells, thereby blocking the CD47/SIRPα don't-eat-me signal. (*e*) A neutrophil engager bispecific antibody, TrisomAb, which recruits neutrophils via CD89 to kill EGFR-expressing tumor cells. Abbreviations: ADCP, antibody-dependent cellular phagocytosis; APC, antigen-presenting cell; BiTE, bispecific T cell engager; EGFR, epidermal growth factor receptor; NK, natural killer; SIRPα, signal-regulatory protein α. Figure adapted from images created with BioRender.

assembled using knobs-into-holes technology (166). CD20 is a surface marker expressed on B cells in all stages of development, except during the precursor and terminal differentiation phases, and thus serves as a good target for many B cell cancers, which are dominated by immature blast cells. The IgG format of mosunetuzumab increases serum half-life over the smaller and Fc-lacking BiTE formats, and these improved pharmacokinetic properties allowed for intermittent dosing, decreasing CRS incidence in clinical studies (166). Mosunetuzumab received accelerated FDA approval in 2022 for treatment of relapsed or refractory follicular lymphoma after two or more lines of systemic therapy (167).

Another example of a clinically approved drug that uses a similar mechanism to BiTEs is teclistamab (Janssen Biotech's Tecvayli®). Teclistamab is a humanized IgG4 bispecific DuoBody antibody (168), which is assembled using controlled Fab-arm exchange. The tumor-specific arm of teclistamab binds B-cell maturation antigen, a receptor that is expressed predominantly on the surface of terminally differentiated B cells and prominently presented in multiple myeloma. Teclistamab showed robust efficacy in clinical trials and was approved for treatment of relapsed and refractory multiple myeloma in 2022 (169). This antibody provides another example in which the mechanism of BiTEs was applied successfully to an IgG format, resulting in superior pharmacokinetic properties and improved safety outcomes.

In addition to IgG formats using BiTE mechanisms, some drugs have similar structure to BiTEs but employ alternative binding domains. One example of this is the clinically approved therapeutic tebentafusp (Kimmtrak®, developed by Immunocore Ltd.). Tebentafusp is an immune-mobilizing monoclonal T cell receptor (TCR) against cancer, a fusion protein consisting of an anti-CD3 scFv linked to a soluble, affinity-enhanced TCR (170). In the case of tebentafusp, the TCR portion of the drug is specific to the gp100 peptide, a melanoma-associated antigen presented on human leukocyte antigen-A*02:01 (171), which is present in approximately 27% of the Caucasian population in the United States (172). Of particular note, due to the engineered picomolar affinity of its TCR portion, tebentafusp mediated clearance of target cells in vitro that expressed as few as 5 to 10 epitopes per cell (173, 174). Tebentafusp showed promising clinical results in patients with metastatic uveal melanoma, culminating in FDA approval in 2022 for the treatment of unresectable or metastatic uveal melanoma (175–177). In this case, the engineering of a soluble TCR instead of an scFv led to a clinically successful drug, establishing an alternative family of molecules to antibody Fv regions for specific targeting of T cell engager antibodies.

In addition to targeting cancer, T cell engagers have been used to treat infectious diseases, such as human immunodeficiency virus (HIV). One strategy for targeting HIV has been to use bispecific antibodies to target latent HIV cell reservoirs (178). For example, diabodies have been designed that combine a heavy chain targeting CD3 with a light chain targeting an HIV-1 envelope protein epitope, thus localizing T cells to HIV-infected cells to effect cell killing (178). The most advanced drug candidate in this category of molecules is MGD020 (developed by MacroGenics), which has

been used alone or in combination with a second diabody (MGD014) in phase 1 clinical trials for individuals with HIV-1 who are taking antiretroviral therapy (179).

4.2. Immune Checkpoint Inhibitor Multispecific Antibodies

As an alternative approach to T cell engagement, antibodies have been employed to block intrinsic immunosuppressive networks within T cells (known as immune checkpoint pathways) to unleash their immunostimulatory potential. Activation of natural T cells is initiated by antigen-dependent activation through binding of the TCR to a peptide antigen presented by a MHC molecule on an antigen-presenting cell (APC). However, the strength and nature of T cell activation are governed by interactions of additional co-stimulatory and co-inhibitory molecules on the surface of T cells and APCs (**Figure 4*b***). Blockade of T cell–suppressive pathways, known as immune checkpoint inhibition, has revolutionized cancer treatment and has shown efficacy in the context of refractory or recurrent tumors for which all other therapies fail. Cytotoxic T lymphocyte antigen-4 (CTLA-4) was the first protein to be clinically targeted by immune checkpoint inhibition. CTLA-4 is expressed on the surface of T cells in lymphatic tissue during the early stages of activation and dampens their activity through competitive binding to and trans-endocytosis of co-stimulatory ligands, as well as through active delivery of inhibitory signals (180–183). The anti-CTLA-4 antibody ipilimumab (Bristol-Myers Squibb's Yervoy®) demonstrated robust antitumor activity in preclinical and clinical studies, leading to FDA approval for treatment of advanced-stage melanoma in 2011 (184, 185), and an additional anti-CTLA-4 antibody (AstraZeneca's tremelimumab, Imjudo®) was approved for treatment of NSCLC and inoperable liver cancer in 2022. Another immune checkpoint protein, PD-1, is expressed on T cells in peripheral tissues and, upon activation by its cognate ligand programmed death-ligand 1 (PD-L1), limits their activity by down-modulating immunostimulatory signaling pathways (186–189). Cancer cells frequently overexpress PD-L1 as a means of evading immune destruction (190). Antibodies that neutralize PD-1 (Merck's pembrolizumab, Keytruda®; Bristol Myers Squibb's nivolumab, Opdivo®; Sanofi/Regeneron's cemiplimab, Libtayo®) and PD-L1 (Roche's atezolizumab, Tecentriq®; Merck's avelumab, Bavencio®; and AstraZeneca's durvalumab, Imfinzi®) have been approved for use in various cancers and have led to significant improvements in disease outcomes (191, 192). These antibodies, when administered as monotherapies, lead to dramatic improvement in durable response rates; however, more than 80% of patients fail to respond to treatment (193, 194). To address this efficacy gap, there have been efforts to combine CTLA-4 and PD-1 blockers. Encouragingly, ipilimumab plus nivolumab combination therapy remarkably enhanced antitumor efficacy in metastatic melanoma patients (195). However, combination antibody therapies require careful dosing optimization and also significantly increase the incidence of toxicities (196). For example, in a phase 3 clinical trial of ipilimumab plus nivolumab combination therapy, 55% of patients reported grade 3 or 4 immune-related adverse events, whereas 16.3% and 27.3% of the patients with grade 3 or 4 immune-related adverse events were found in the nivolumab and ipilimumab monotherapy groups, respectively (197). To overcome these obstacles and improve the efficacy of cancer immunotherapy, Jiangsu Alphamab developed KN046, a single-domain antibody–based humanized bispecific antibody that targets both PD-L1 and CTLA-4 and contains an Fc domain (198). In a phase 2 study in patients with metastatic NSCLC who failed platinum-based chemotherapy, KN046 showed promising efficacy as a second-line treatment while simultaneously reducing side effects and treatment costs because of its simplified structure (199).

In addition to PD-1 and CTLA-4, T cells express additional inhibitory surface receptors that have potential as targets in cancer immunotherapy (185), including lymphocyte-activation gene 3 (LAG-3) and T cell immunoglobulin and mucin-domain containing-3 (TIM-3) (200). LAG-3 is

expressed on activated T, B, and NK cells but also on plasmacytoid DCs (201). LAG-3 binds to MHC class II and competitively inhibits their binding to TCR and CD4, thus suppressing $CD4^+$ T cell–mediated immunity (201, 202). TIM-3 is constitutively expressed on innate immune cells such as monocytes/macrophages, DCs, mast cells, and mature NK cells and binds to several ligands, including galectin-9, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1), phosphatidylserine, and high-mobility group box-1 protein (HMGB1) (203). Each ligand mediates a unique mechanism of immunosuppression: Galectin-9 interaction induces apoptosis in TIM3-expressing T cells (204); CEACAM-1 and TIM-3 form a heterodimer on the surface of activated T cells to downregulate their function (205); phosphatidylserine on apoptotic cells interacts with TIM-3 on the surface of macrophages and DCs to promote phagocytosis and cross-presentation, thereby preventing autoimmune activity (206); and HMGB1 interaction with TIM-3 on myeloid cells interferes with HMGB1-mediated immune activation (207). Preclinical mouse models have demonstrated that blocking the PD-1/PD-L1 pathway can lead to upregulation of LAG-3, TIM-3, or other immune checkpoint proteins as a compensatory mechanism, and these data have inspired combination immunotherapy approaches. Combining the anti-PD-1 antibody nivolumab and the anti-LAG3 antibody relatlimab (developed by Bristol Myers Squibb) led to promising clinical benefits in melanoma patients who were unresponsive to prior anti-PD-1/PD-L1 therapy (201), leading to FDA approval of this dual antibody combination (Opdualag™). Anti-TIM-3 antibodies, including Tesaro's TSR-022 (NCT02817633), Novartis's MBG453 (NCT02608268), and Lilly's LY3321367 (NCT03099109), have been studied in first-in-human phase 1/2 clinical trials. Many of these anti-TIM-3 antibodies are being investigated in combination with anti-PD-1/PD-L1 antibodies, and preliminary data have shown that this combination is broadly safe and well tolerated (208). In addition to combination antibody therapies, new approaches are focusing on development of bispecific antibodies that target multiple immune checkpoint proteins. LB1410, a recombinant humanized anti-PD-1/TIM-3 bispecific antibody developed by L&L Biopharma Co., Ltd., simultaneously blocks PD-1- and TIM-3-mediated immunosuppressive signaling, which led to improved antitumor efficacy compared to combination treatment with anti-PD-1 and anti-TIM-3 antibodies in preclinical studies (209). Additionally, AK129, a humanized IgG1 bispecific antibody targeting PD-1 and LAG-3 developed by Akeso, showed promising preclinical results in T cell activation and in vivo mouse cancer models compared to combination antibody treatment (210). Collectively, these examples highlight the advantages of bispecific antibodies over monospecific antibody combination therapies in immune checkpoint inhibition.

4.3. Immune Checkpoint Agonist Multispecific Antibodies

Full activation of natural T cells requires two signals: (*a*) the aforementioned interaction between a TCR on the T cell surface and a peptide loaded in an MHC molecule on the APC surface and (*b*) the interaction between a co-stimulatory receptor expressed on the T cell and its cognate ligand on the APC (211) (**Figure 4*b***). In particular, CD28 is a key co-stimulatory receptor that signals through recognition of CD80 and CD86 (also known as B7 ligands) on APCs (212, 213). Mounting evidence has established the use of agonistic antibodies targeting co-stimulatory receptors such as CD28 as a promising strategy to achieve antitumor activity (214).

The concept of CD28 superagonism was originally discovered through the observation that certain rat CD28-specific monoclonal antibodies could activate T cells in the absence of TCR signaling (212). The injection of a CD28 superagonist antibody into rats resulted in preferential activation and expansion of regulatory T cells (Tregs). Building on these findings, and given Tregs' central role in regulating autoimmunity, Treg activation by CD28 superagonists might be an effective novel treatment strategy for autoimmune diseases such as rheumatoid arthritis, multiple

sclerosis, Guillain–Barré syndrome, or type 1 diabetes (213). TeGenero generated a CD28-specific fully humanized IgG4 superagonist antibody (TGN1412). The IgG4 isotype was used because of its limited ability to recruit immune effector functions, which the investigators hypothesized would reduce toxicity (215). A first-in-human trial of TGN1412 was conducted in six healthy young volunteers. Unexpectedly, all volunteers experienced CRS with multiple organ failure following a single intravenous dose of the drug (213, 216). Within 90 min, all subjects exhibited a systemic inflammatory response by rapid induction of proinflammatory cytokines, followed by headache, myalgias, nausea, diarrhea, erythema, vasodilatation, and hypotension, and within 24 h, severe depletion of lymphocytes and monocytes was observed. The participants were hospitalized and received intensive cardiopulmonary support, high-dose methylprednisolone, and an anti-IL-2Rα antagonist antibody; thankfully, all patients survived (217). It was likely that CD28 superagonism inappropriately activated effector T cells, leading to the observed explosive release of multiple proinflammatory cytokines, including tumor necrosis factor α, interferon γ, IL-2, and IL-6, all of which were found at skyrocketing levels in blood samples taken a few hours after drug administration (213). Due to this catastrophic clinical failure, TGN1412 development was terminated, and TeGenero went into insolvency. However, TheraMAB later inlicensed TGN1412, renamed it TAB08, and resumed preclinical development. A healthy volunteer trial in 2011 started dosing at 0.1 μg/kg (1,000-fold lower than the original clinical study), and the dose was gradually escalated to a maximum of 7 μg/kg, still well below the original trial dosing in 2006 (100 μg/kg) (218). No proinflammatory cytokine release was observed, even at the highest doses employed, and clinical trials are underway in patients with rheumatoid arthritis, systemic lupus erythematosus, and solid tumors.

Building on initial work with monospecific co-stimulatory antibody agonists, several bispecific antibodies have been developed for controlled activation of CD28. Examples include an anti-PD-L1/CD28 bispecific antibody that demonstrated enhanced T cell degranulation, cytokine secretion, and cancer cell cytotoxicity in combination with CD3 stimulation only in the presence of target antigen (219). Indeed, in human CD28 knock-in mice bearing MC38 colorectal cancer tumors expressing human PD-L1, anti-PD-L1/CD28 bispecific antibodies suppressed tumor growth greater than the monospecific anti-PD-L1 antibody (219). In another instance, a bispecific antibody targeting CD20 and CD28 induced T cell activation in peripheral blood mononuclear cell cultures from patients with chronic lymphocytic leukemia and potently killed $CD20^+$ tumor cells (220). These tumor-targeted bispecific antibodies represent a promising approach toward improving tumor selectivity, efficacy, and safety of immune antagonists in target antigen-overexpressing malignancies.

In addition to CD28, 4–1BB (CD137, TNFR superfamily 9) is another co-stimulatory receptor expressed on activated T and NK cells. The interaction of 4–1BB and its major ligand, 4–1BB ligand (4–1BBL), positively triggers a signaling cascade that enables upregulation of antiapoptotic molecules, cytokine secretion, and enhanced effector function (221). Due to its upregulated expression in the tumor microenvironment and its association with tumor progression, 4–1BB is a promising target for immunotherapy (222). In preclinical studies, 4–1BB agonist antibodies showed antitumor efficacy in several solid tumor models (223, 224). In a CT26 colorectal cancer mouse model, a 4–1BB agonist demonstrated dose-dependent suppression of tumor proliferation (214). Encouraged by this preclinical study, researchers developed fully human antibodies against 4–1BB, including urelumab (Bristol Myers Squibb's BMS-663513) and utomilumab (Pfizer's PF-05082566) (194, 225). Urelumab, a fully human IgG4 monoclonal antibody, became the first 4–1BB-targeted therapeutic to enter a phase 1/2 trial and was investigated for treatment of advanced or metastatic cancers. A high frequency of adverse effects, including fatigue, reversible grades 3–4 transaminitis, and grades 3–4 neutropenia, was observed, and a phase 2 trial was

terminated due to a high incidence of grade 4 hepatotoxicity (226). In contrast, utomilumab, a fully human IgG2 antibody against 4–1BB, was studied in a phase 1 monotherapy trial of patients with advanced solid tumors or lymphoma and was found to be well tolerated, with none of the patients experiencing liver toxicity (227). Utomilumab in combination with mogamulizumab was evaluated in a phase 1b study (NCT02444793), and the results showed that the combined treatment in patients with advanced solid tumors was safe and tolerable (228). Utomilumab is currently in phase 3 trials for diffuse large B cell lymphoma.

In an effort to localize the effects of 4–1BB agonists, bispecific antibodies targeting disease-associated antigens in combination with 4–1BB represent a growing class of therapeutic candidates. Some bispecific antibodies, including the full-length IgG1 anti-PD-L1/anti-4–1BB bispecific antibody GEN1046 (developed by Genmab/BioNTech) and the anti-HER2/anti-4–1BB bispecific antibody PRS343, which is composed of an alternative binding scaffold (Anticalin®) targeting 4–1BB linked to a monospecific antibody specific for the HER2 receptor (developed by Pieris Pharmaceuticals), are currently being evaluated in clinical trials (229, 230). Additionally, ES101 (INBRX-105), a tetravalent bispecific antibody targeting PD-L1 and 4–1BB developed by Inhibrx, is composed of four domains, two of which target PD-L1, whereas the other two target 4–1BB (231).

Beyond bispecific formats, trispecific and tetraspecific immune agonist antibodies also have been developed. NM21–1480, developed by Numab Therapeutics AG, is a trispecific antibody targeting PD-L1, 4–1BB, and human serum albumin (HSA) (232), which combines the synergistic effects of immune checkpoint blockade, signal 2 agonism, and human serum albumin–mediated serum half-life extension in a single cancer therapeutic. Systimmune has developed two tetraspecific antibodies (formatted as three serial scFvs in an IgG framework) as potential cancer drugs: GNC-035, which targets PD-L1, 4–1BB, CD3, and ROR1, and GNC-039, which targets PD-L1, 4–1BB, CD3, and EGFR. Overall, although challenges remain for immune co-stimulatory agonist antibodies, such as inadequate T cell infiltration and activation, APC dysfunction, the suppressive tumor microenvironment, and immune-related adverse events, these molecules represent powerful candidates for next-generation therapeutic design.

4.4. Engaging Innate Immune Cells with Multispecific Antibodies

Recruitment of cytotoxic T cells has been the most widely used approach for immune cell–engaging multispecific antibodies. However, toxicity concerns, including CRS and neurotoxicity, remain as therapeutic challenges for this approach (233, 234). As a result, there is growing interest in the development of multispecific antibodies that can engage innate rather than adaptive immune cells as an alternative or complementary strategy to potentially increase the therapeutic index for immune-interfacing antibodies in disease treatments. Innovative strategies based on NK cells have gained tremendous traction due to the unique features of NK cell biology, specifically their human leukocyte antigen–independent recognition of distressed cells, such as tumor cells and virally infected cells (235–237). The initiation of NK cell cytolytic activity is strictly regulated by a set of integrated signals derived from both stimulatory and inhibitory receptors on the cell surface (237–239). NK cells' ability to distinguish target cells in distress from normal healthy cells through the presence or absence of certain surface proteins has been harnessed therapeutically through the development of multispecific antibodies designed for NK cell recruitment (18, 238, 240). Several stimulatory receptors on NK cells, such as CD16a (FcγRIIIA, found in several innate immune cells), NK group 2D (NKG2D), the signaling lymphocytic activation molecule family members, and natural cytotoxicity receptors, have been evaluated for bispecific engagement (85–87). For example, AFM13, developed by Affimed Therapeutics, is a tetravalent, bispecific innate immune cell engager that can recruit NK cells via CD16A to interface with

CD30-expressing tumor cells (**Figure 4*c***). In a phase 1b/2a study, AFM13 demonstrated a safe and well-tolerated pharmacokinetic profile in pretreated patients with relapsed or refractory lymphoma and showed an impressive 50% objective response rate (241–243). Unfortunately, only a modest response rate to AMF13 was observed in a phase 2 monotherapy study (244); however, the NK-recruiting mechanism suggested the potential for combination therapies. Indeed, combination of AMF13 with either pembrolizumab or preactivated cord blood–derived NK cells showed promising preliminary clinical results (245–247). Several other NK cell engagers, such as Innate Pharma's IgG-based IPH6101/SAR'579 (anti-NKp46/CD16/CD123) (NCT05086315) and GT Biopharma's trispecific scFv GTB-3550 (anti-CD16/CD33 fused to IL-15) (247), are also under clinical evaluation (240, 248).

Another attractive approach for innate immune cell engagement that is under active exploration is the recruitment of macrophages to coordinate ADCP for disease treatment. Macrophages exert a high degree of plasticity in response to environmental cues, and their phagocytic activities can be modulated through potentiation or inhibition of checkpoint proteins expressed on their surface (249–252). CD47, which is broadly expressed across different cell types, is the best studied phagocytic checkpoint protein to date. CD47 interacts with signal-regulatory protein (SIRP)α, which is found on all myeloid cells, including macrophages, providing a don't-eat-me signal to protect against elimination of healthy cells that express CD47 (253). Thus, the design of multispecific antibodies that can simultaneously block the CD47/SIRPα interaction to activate phagocytosis of diseased cells and also disrupt signaling through a disease-associated antigen or other inhibitory immune checkpoint protein, such as PD-1, represents a promising therapeutic approach. TG-1801, developed by TG Therapeutics, is an anti-CD47/anti-CD19 bispecific κλ body that induces potent ADCC and ADCP of $CD19^+$ lymphoma cells (62, 254) (**Figure 4*d***). A phase 1 study of TG-1801 showed a relatively safe and well-tolerated pharmacokinetic profile either as a monotherapy or in combination with TG Therapeutics's anti-CD20 antibody ublituximab (Briumvi®) (255). Several examples of CD47-based bispecific antibodies, such as ImmunoOnco's IMM0306 (anti-CD47/CD20), NovImmune's NI-1801 (anti-CD47/anti-MSLN), Innovent Biologics's IBI322 (anti-CD47/anti-PD-L1), and HanX Biopharmaceuticals's HX009 (anti-CD47/anti-PD-L1), are also currently undergoing clinical studies in either phase 1 or 2 trials (1, 254, 256–259).

Neutrophils have been long regarded as front-line defenders for the immune system, and only relatively recently were neutrophils recognized to play important roles in diseases beyond acute inflammation, including autoimmune diseases, cancers, and other chronic inflammatory diseases (260–262). Similar to NK cells and macrophages, neutrophils also express FcγRs, including activation-gated expression of the high-affinity FcγRI (CD64), to mediate ADCC (263). Thus, several FcγRI-targeted bispecific antibodies have been exploited to recruit neutrophils to treat cancer, including Medarex's MDX-H210 (anti-FcγRI/anti-HER2) and MDX-447 (anti-FcγRI/anti-EGFR). However, because no significant antitumor activity was observed in either phase 1 or 2 clinical trials, investigation of these molecules was discontinued (263–265). Targeting IgA Fc receptor FcαRI (CD89), which is also expressed on neutrophils, has exhibited a more potent tumoricidal capacity through the release of neutrophil attractant leukotriene B4 (266) (**Figure 4*e***). TrisomAb is a bispecific antibody with one arm that targets either EGFR or gp75 and a second arm that recruits neutrophils through an anti-FcαRI antibody (267). TrisomAb led to effective eradication of tumor cells in vivo, whereas depletion of either neutrophils, NK cells, or macrophages significantly reduced the therapeutic activity, indicating that both neutrophil recruitment and Fc effector function were critical to activity (267). Another bivalent, bispecific antibody, termed gremubamab (AstraZeneca's MEDI3902), was explored for treatment of *Pseudomonas aeruginosa* infection (268–270). Despite its well-tolerated safety profile, gremubamab did

not reduce the risk of nosocomial *P. aeruginosa* pneumonia in *P. aeruginosa*–colonized mechanically ventilated patients in a phase 2 clinical trial; thus, further investigation is required to evaluate its therapeutic potential (270).

5. CONCLUSION

Multispecific antibodies are a rapidly growing class of therapeutics that enable targeted treatment of complex diseases through various mechanisms. One important strategy for multispecific antibodies is targeting multiple disease markers simultaneously. Many applications of multispecific antibodies have focused on cancer, wherein targeting signaling pathways and tumor-associated antigens have shown clinical success. More recently, this strategy has been used in diseases beyond cancer, such as hemophilia A, where simultaneous engagement of multiple targets is key to therapeutic success. Another powerful strategy for multispecific antibodies is use of multiparatopic architectures, which engage multiple sites on the target molecule to manipulate receptor trafficking. Multiparatopic antibodies also have been exploited for intracellular delivery of cytotoxic payloads, for example, in ADC formats. Another application for multiparatopic antibodies is to improve binding affinity and neutralization potency in targeting infectious diseases, such as the SARS-CoV-2 virus. Alternatively, multiparatopic antibodies can serve as receptor agonists, wherein clustering and crosslinking help boost cellular responses to receptor binding. In addition to targeting mechanisms present in pathogenic cells and viruses, multispecific antibodies have been used to engage immune cells and bring them in proximity to disease targets of interest. One of the largest classes of immune-interfacing multispecific antibodies is T cell engagers, which use an anti-CD3 domain to stimulate T cell activity. Multispecific antibodies have also been used to either block immunosuppressive checkpoint pathways or agonize co-stimulatory molecules, in both cases leading to enhanced T cell activation. Immune-interfacing antibodies have also been developed to engage innate immune cells. One of the primary targets has been NK cells, although emerging efforts focus on recruitment of macrophages and neutrophils.

Although many of the multispecific antibodies discussed herein are currently at the preclinical stage, the recent and rapid increase in clinically approved bispecific antibodies and the large uptick in clinical trials of multispecific molecules demonstrate expanding interest in this category of drugs. Given recent advancements in molecular design and the development of innovative tools for protein engineering, we have reached an exciting juncture with access to a much larger range of antibody topologies and antigen specificities. Moreover, manufacturing progress has made the development of multispecific antibodies more attainable than ever before. Overall, multispecific antibodies offer exciting new opportunities and a vast repertoire of synergistic mechanisms to solve complex medical problems that have eluded monospecific antibodies and small-molecule drugs.

DISCLOSURE STATEMENT

J.B.S. is a cofounder of AbMeta Therapeutics, which focuses on bispecific antibody drug development. J.F., B.M., and J.T. are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors acknowledge funding from an Allegheny Health Network-Johns Hopkins Research Award (to J.B.S.), a Gabrielle's Angel Foundation award (to J.B.S.), a National Science Foundation CAREER award (2143160 to J.B.S.), and a National Institutes of Health (NIH) grant, R21 CA256084 to J.B.S. J.F. is supported by NIH T32 GM135131. All figures were created with BioRender.com.

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