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A review of bispecific antibodies and antibody constructs in oncology and clinical challenges

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ABSTRACT

Bispecific antibodies (bsAbs) are antibodies that bind two distinct epitopes to cancer.. For use in oncology, one bsAb has been approved and 57 bsAbs are in clinical trials, none of which has reached phase 3. These bsAbs show great variability in design and mechanism of action. The various designs are often linked to the mechanisms of actions. The majority of bsAbs engage immune cells to destroy tumor cells. However, some bsAbs are also used to deliver payloads to tumors or to block tumor signaling pathways. This review provides insight into the choice of construct for bsAbs, summarizes the clinical development of bsAbs in oncology and identifies subsequent challenges.

Contents

1. Introduction

Advances in biotechnology leading to improved antibody production and recombination techniques have fueled the development of antibodies and myriad antibody constructs. Currently, 72 antibodies are approved by the Food and Drug Administration (FDA) of which 30 are registered for the treatment of cancer patients (TheAntibodySociety, 2018). Antibodies are playing an increasing role in cancer treatments (Sliwkowski & Mellman, 2013). The understanding of antibodies and how to modify their pharmacokinetic and physicochemical properties has grown (Jain, Kamal, & Batra, 2007). After being established as standard treatments, increasingly complex antibody constructs have been developed (Carter & Lazar, 2017). Besides intact immunoglobulin

Abbreviations: ALL, Acute lymphoblastic leukemia; AML, Acute myeloid leukemia; BiTE, Bispecific T cell engager; bsAb, Bispecific antibody; CEA, Carcinoembryonic antigen; DART, Dual affinity retargeting; DR5, Death receptor 5; EDV, Engeneic delivery vehicle; EGFR, Epidermal growth factor receptor; EMA, European Medicines Agency; EpCAM, Epithelial cell adhesion molecule; FAP, Fibroblast activation protein; FDA, Food and Drug Administration; HER2, Human epidermal growth factor receptor 2; IgG, Immunoglobulin G; LAG-3, Lymphocyte activation gene 3; MET, Mesenchymal epithelial transition factor; NK cell, Natural killer cell; PBMC, Peripheral blood mononuclear cells; PD-1, Programmed cell death 1; PD-L1, Programmed death ligand 1; scFv, Single chain variable fragment; TAA, Tumor-associated antigen; TCR, T cell receptor; Treg, Regulatory T cells; VEGF-A, Vascular endothelial growth factor-A.

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G (IgG) antibodies, the first antibody drug conjugates and bispecific antibodies (bsAb) have been approved for the treatment of cancer patients, and other antibody constructs are in clinical trials (Carter & Lazar, 2017) (Fig. 1).

Standard human antibodies are monospecific antibodies in which both binding sites are directed against the same target. A bsAb is a more complex construct in which the binding sites are directed to different targets. This enables novel and unique mechanisms of actions (Chames & Baty, 2009; Fan, Wang, Hao, & Li, 2015) such as engaging immune cells to tumor cells, delivering payloads to tumors, and blocking signaling important for the tumor (Fig. 2). Each mechanism of action can require pharmacokinetic properties that can be obtained by modifying the bsAb. An abundance of preclinical data has been published about these bsAb constructs and their mechanisms of action (Brinkmann & Kontermann, 2017).

In oncology, two bsAbs have been approved for use in the clinic. Catumaxomab, targeting Epithelial cell adhesion molecule (EpCAM) and CD3, was approved by the European Medicines Agency (EMA) in 2009 for the treatment of malignant ascites (Seimetz, Lindhofer, & Bokemeyer, 2010). However, at the request of the marketing authorization holder market authorization was withdrawn in June 2017. Blinatumomab, targeting CD19 and CD3, was approved by the FDA in December 2014 and by the EMA in December 2015 for the treatment of Philadelphia chromosome negative B cell acute lymphoblastic leukemia (ALL) (Przepiorka et al., 2015). Outside of oncology the bsAb emicizumab, which binds clotting factors IXa and X, was approved by the FDA in November 2017 and by the EMA in March, 2018 for the treatment of hemophilia A.

Currently, 57 bsAbs, including blinatumomab, are in clinical trials in cancer patients (Table S1) of which 38 use the same mechanism of action: engagement of immune cells with tumor cells. Of the remaining 19 bsAbs in clinical trials, five deliver a payload to tumors and 14 are blocking signaling in the cancer environment.

This review has two aims: 1) to summarize the ongoing clinical development of bsAbs in oncology by evaluating their choice of construct, and 2) to identify the challenges bsAbs are facing in this clinical development.

2. Search strategy

Articles published in English until September 5 2018 were searched using PubMed. The search strategy was based on the terms bispecific antibody, T cell engager, immune cell engager, antibody constructs, targeted delivery and variations of these terms.

The ClinicalTrials.gov database was searched for trials evaluating bsAbs until September 5 2018, based on the abovementioned terms and the names of known bsAbs found in literature. BsAbs were considered to be approaching the clinic if their clinical trials were not all terminated, withdrawn or completed before 2014 without reporting results. Additionally, bsAbs were also excluded when press releases stated that their development had ceased.

Registered drugs were verified on FDA.gov and ema.europe.eu. Reference lists of articles were manually searched for relevant articles missed in the PubMed or ClinicalTrials.gov searches.

3. Bispecific antibody formats and modifications

3.1. Antibody format

An antibody consists of heavy and light domains that connect to form chains. Light chains consist of two light domains and heavy chains of four heavy domains. A light and heavy chain together form a pair, and two heavy-light chain pairs comprise an antibody (Fig. 1A). The region where the two pairs connect is called the hinge region. IgG is the most abundant antibody in the blood and it is the backbone most often used for antibody therapeutics. Endogenous IgGs have small variations in their hinge regions, resulting in IgG subtypes (Irani et al., 2015).

An antibody can be also divided into functional parts: the tail (Fc region) and the binding sites (Fab regions). The Fc region mediates the effector functions that lead to immune-mediated target-cell killing (Scott, Wolchok, & Old, 2012). The Fc region can also be recognized by a receptor called the neonatal receptor, which is involved in regulating the IgG serum levels and actively prolongs the biological half-life (Roopenian & Akilesh, 2007). This process is called neonatal recycling. Connected to the Fc region are the Fab regions containing the variable fragments that make up the binding sites.

3.2. Producing bsAbs

The two binding regions of an antibody target the same epitope. An antibody is therefore bivalent but monospecific. In contrast, bsAbs that

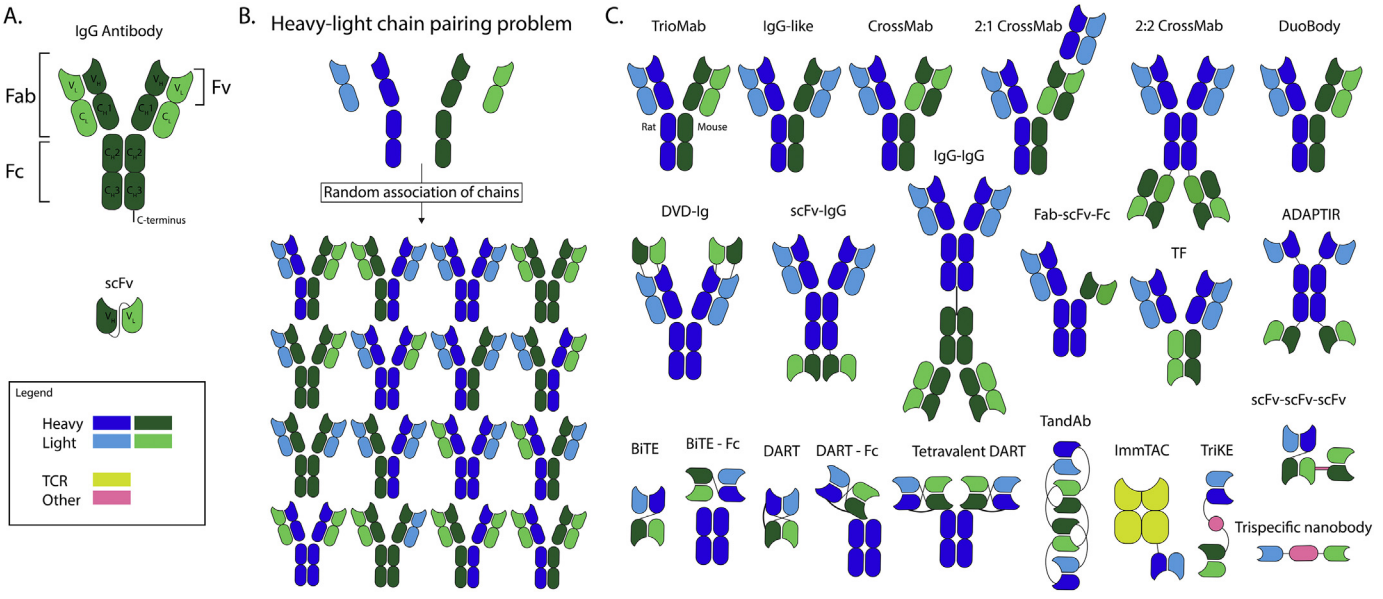

Fig. 1. Schematic overview of the antibody structure and bsAb constructs currently being evaluated in clinical trials. (A) The IgG antibody construct consists of Fab and Fc regions. The binding part of the Fab region is called the single chain variable fragment (scFv). The antibody exists of two heavy chains (V_H and C_H) and two light chains (V_L and C_L). These chains can be subdivided by variable (V_H and V_L) and constant domains (C_H and C_L). (B) Random heavy-light chain pairing. Two possibilities yield the desired outcome. (C) BsAb constructs currently approved or in clinical trials.

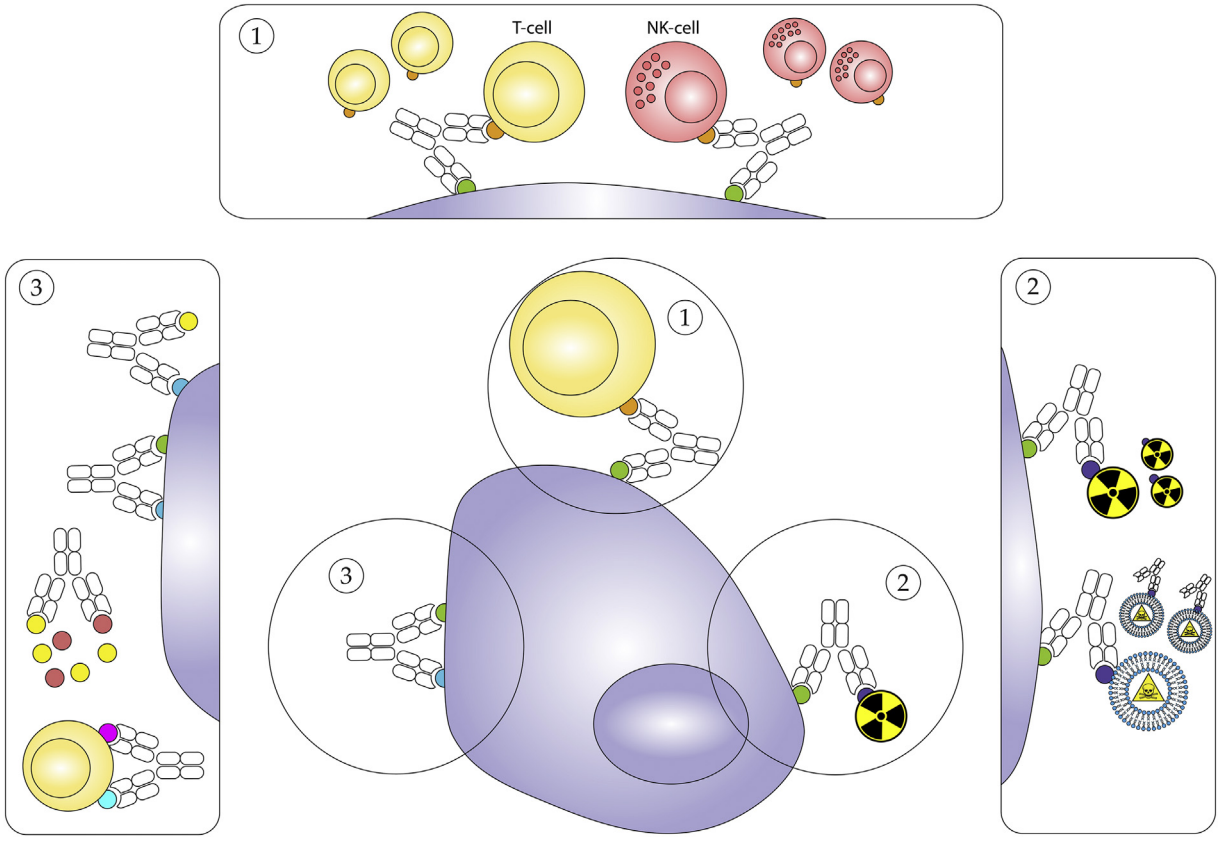

Fig. 2. Simplified schematic overview of the proposed mechanisms of action for bispecific antibodies (bsAbs) in clinical trials for oncology. 1. Engagement of immune cells to the tumor cell. Immune cells can be engaged to tumor cells. 2. Targeted delivery of payloads. Tumor cells are being targeted with a bsAb having affinity for both the tumor and a payload. 3. Blocking signaling. Two targets are being disrupted by the bsAb.

have affinities for two different epitopes bind to two targets, either monovalently or bivalently depending on the construct. Antibodies are generally produced from hybridoma cell lines, which are a fusion of an antibody-secreting B cell and an immortal myeloma cell line (Köhler & Milstein, 1975). BsAbs can be produced by fusing two hybridoma cell lines to form a quadroma, which results in a mixture of IgG molecules (Jain et al., 2007). They can also be produced by conjugating two existing antibodies or their fragments. Another option, which is popular for its flexibility, is using recombinant proteins. Using genetically engineered recombinant proteins creates options regarding origin, composition, and production system (Kontermann, 2012). For example, such proteins can be used to control the association of heavy and light chains. A basic bsAb comprises one heavy-light chain pair from one antibody and another heavy-light chain pair from another antibody. When the four individual chains are combined, they associate randomly, and 16 combinations of IgG molecules can arise. Two of those combinations result in the desired bsAbs with a heterodimerized heavy chain bound to their specific light chains stemming from the same antibody (Fig. 1B). Chimeric quadromas, common light chains and recombinant proteins can provide solutions by limiting the options for association. Chimeric quadromas have species-restricted heavy-light chain pairing. Moreover, using common light chains also prevents undesired heavy-light chain association. Recombinant proteins can force the correct association of heavy-light chains and the heavy chains by multiple means. Examples are the knob-in-holes approach where one heavy chain is engineered with a knob consisting of relatively large amino acids and the other heavy chain is engineered with a hole consisting of relatively small amino acids (A. M. Merchant et al., 1998). Other examples are the constructs with their fragments connected by peptide chains, such as bispecific T cell engagers (BiTE) molecules, thereby circumventing random association of the chains (Mack, Riethmuller, & Kufer, 1995).

3.3. Rational design

Like an antibody, a bsAb can be modified in countless ways to customize its functionality and enhance its efficacy, such as by modulating the immunogenicity, effector functions and half-life of an antibody (Brinkmann & Kontermann, 2017; Carter, 2006).

As regards modulating the immunogenicity, the immunogenic parts of antibody constructs that arise from production in mice are often replaced by human counterparts to reduce auto-immunogenicity (Birch & Racher, 2006; Khazaeli, Conry, & LoBuglio, 1994). This results in the production of chimeric and humanized antibody constructs. Fully human antibody constructs are increasingly being produced, usually by phage display or by immunizing mice that are transgenic for human IgG (Carter, 2006). With phage display, a library of phages expressing antibody parts is screened for affinity to an antigen. Other parts of antibody constructs that can elicit immunogenicity are foreign amino acid sequences, possibly introduced by novel protein engineering (Tovey & Lallemand, 2011).

As regards the effector function of an antibody, the Fc region plays a central role in mediating this process. The region is involved in the immune-mediated cell-killing mechanisms such as complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (Scott et al., 2012). In contrast to tumor-cell targeting antibodies, for which a functional Fc region is desired for target cell killing, antibodies binding immune cells are designed to mitigate this cell killing. The immune-mediated cell-killing mechanisms can be influenced by glycoengineering and changing the amino acid sequence of the Fc region (Jiang et al., 2011; Shields et al., 2001). These techniques can enhance or diminish the immune-mediated cell killing via the antibody, depending on the location and the function of the glycans and the amino acids of the antibody that are modified. Besides abolishing

immune-mediated cell killing, the entire Fc region can also be deleted, leading to the distinction between Fc region-bearing and Fc region-lacking antibodies (Kontermann & Brinkmann, 2015). This elimination also drastically reduces the size of an antibody which affects pharmacokinetics including its clearance and tumor penetration (Schmidt & Wittrup, 2009).

An intact IgG antibody is 150 kDa and is cleared by the liver, while proteins with a molecular weight below <60 kDa are cleared by the kidneys. Renal clearance is faster than hepatic clearance (Wittrup, Thurber, Schmidt, & Rhoden, 2012). The size of an antibody can also be altered by removing domains in the non-binding region of the Fab-region, the C_L and C_H1 domains (Fig. 1A). If the non-binding domains are deleted from the construct only the essential binding sites, i.e. the variable fragments remain. These variable fragments linked together by a single peptide chain are called a single chain variable fragment (scFv) (Weisser & Hall, 2009). ScFvs are cleared rapidly from the circulation due to their small size and the lack of the neonatal receptor. Therefore, continuous administration of scFvs may be necessary when a constant blood level is required for treatment of patients (Portell, Wenzell, & Advani, 2013). Moreover, scFvs can serve as building blocks to create bsAbs (Fig. 1C).

Besides increasing the size, others options to extend the half-life of an antibody construct are fusing with or binding to albumin, conjugating to polyethylene glycol fragments and fusing a Fc region to the construct (Kontermann, 2016). Several bispecific constructs when fused to human serum albumin, show increased in half-life in mouse models (Müller et al., 2007). Also, adding a Fc region to bispecifics can circumvent the continuous administration that is required for small constructs due to rapid clearance (L. Liu et al., 2017; Lorenczewski et al., 2017; Moore et al., 2018). In non-human primates, the serum half-life of various BiTEs was extended from 6 to 44–167 h by fusing Fc region to them (Arvedson et al., 2017).

BsAbs, in contrast to the standard antibody, do not always bind bivalently to one target. Bivalent binding increases the avidity and can affect the pharmacodynamics of the construct. Bivalent antibodies can induce antibody-dependent dimerization. One example is the development of an antibody that blocks mesenchymal epithelial transition factor (MET) kinase signaling. A monovalent antibody was engineered to prevent dimerization of the MET receptors and downstream activation (M. Merchant et al., 2013). Bivalent antibodies targeting CD3 can also induce crosslinking between T cells leading to T cell lysis (Wong, Eylath, Ghobrial, & Colvin, 1990). In contrast, a one-armed antibody targeting CD3 failed to induce T cell lysis in vitro (Wong et al., 1990). To prevent rejection in patients receiving a renal transplant, a bivalent antibody targeting CD3 depleted T cells but also provoked serious cytokine release (Gaston et al., 1991). With immune cell-engaging bsAbs in oncology, immune cell depletion is not desired, so most of these bsAbs bind CD3 monovalently.

4. Engagement of immune cells

The growing interest in cancer immunotherapy is also driving the development of immune cell engaging bsAbs (Wu & Cheung, 2018).

The bsAb blinatumomab engages immune cells to B cell ALL (Kantarjian et al., 2017). It engages the immune cell with the CD3 antigen, a general marker of T cells. The T cell is bound to the tumor by targeting a tumor-associated antigen (TAA). For blinatumomab this TAA is CD19, a marker of B cells. Generally, a TAA should be specific for tumor cells, leaving healthy tissue unharmed. The TAA does not have to play a role in the pathogenesis of the cancer; its primary role in case of immune cell-engaging bsAbs is to provide a binding place at the tumor cell membrane.

The use of immune cell-engaging bsAbs has been explored for over 30 years (Songsivilai & Lachmann, 1990; Staerz, Kanagawa, & Bevan, 1985). Recently, blinatumomab has confirmed the potential of immune cell-engaging bsAbs for the treatment of hematological malignancies (Kantarjian et al., 2017; Topp et al., 2015). In a randomized study, patients with heavily pretreated B cell precursor ALL treated with blinatumomab had a median survival of 7.7 months compared to 4.0 months for the chemotherapy treated group (Kantarjian et al., 2017) (Table 2).

Most bsAbs in clinical trials are immune cell-engaging; 38 of the 57 oncology-related bsAbs reported on ClinicalTrials.gov are of this type (Fig. 3).

4.1. CD3+ T cell-engaging bsAbs

Of the 38 immune cell-engaging bsAbs found in clinical trials, 36 engage T cells by binding to T cell receptor CD3: 18 target hematological malignancies and the remaining 16 target solid cancers.

When both T cell and tumor cell are bound by the bsAb, a cytolytic synapse is formed. In this cytolytic synapse the T cell releases the poreforming perforin and cytotoxic granzyme-B, leading to killing of the target cell, as was proven in vitro (Offner, Hofmeister, Romaniuk, Kufer, & Baeuerle, 2006) and has been visualized by confocal microscopy (Haas et al., 2009). Binding to a T cell in the absence of a target cell does not activate the T cell as shown in in vitro T cell activation and cytotoxicity assays with human peripheral blood mononuclear cells (PBMCs) and BiTEs (Amann et al., 2009; Brischwein et al., 2007).

However, when epidermal growth factor receptor (EGFR) positive and negative cancer cells were mixed in vitro and used to create human xenograft mouse models, a BiTE binding CD3 and EGFR also induced killing in the EGFR-negative cells (Ross et al., 2017). This illustrated that BiTE treatment can provoke killing of non-TAA expressing tumor cells as well.

Preclinical research has suggested the involvement of immune checkpoints in mitigating the response to immune cell-engaging bsAbs in hematological cancers. Addition of AMG330, a BiTE targeting CD33 and CD3, to a co-culture of primary acute myeloid leukemia (AML) cells and PBMCs collected from patients resulted in upregulation of programmed death ligand 1 (PD-L1) on predominantly AML cells (Krupka et al., 2016). Addition of anti-PD-1 and/or anti-PD-L1 antibody enhanced lysis of AML cells in these patient samples(Krupka et al., 2016). In cynomolgus monkeys, a CD3 and B cell lineage marker FcRH5 targeting full-length bsAb for the treatment of multiple myeloma induced PD1 + CD8+ T cells measured in blood, spleen, lymphnodes and bone marrow and depleted their B cells (Li et al., 2017). Combining this bsAb with an anti-PD-L1 antibody in vitro increased lysis of tumor cells transfected with a PD-L1 encoding plasmid (Li et al., 2017).

In many solid tumor mouse models, with functional immune systems, tumor responses have been observed with immune cell-engaging bsAbs (Yu et al., 2017). For these studies, a broad range of TAAs were chosen, including established tumor markers such as carcinoembryonic antigen (CEA), EpCAM, human epidermal growth factor receptor 2 (HER2) and EGFR. However, clinical efficacy data on immune cell-engaging bsAbs in solid cancers in humans is scarce (Table 2).

A noteworthy bsAb is IMCgp100, which engages CD3 to glycoprotein100 (gp100), an antigen associated with melanoma. The construct used for IMCgp100, ImmTAC, targets the surface protein gp100 with a T cell receptor (TCR) instead of the Fab region of an antibody (Liddy et al., 2012) (Fig. 1C). The use of TCRs can enable targeting of intracellular oncoproteins presented by major histocompatibility complex molecules. However, a polyclonal T cell response, such as that generated by CD3-engaging bsAbs, is precluded. A TCR specific for the intracellular WT1 protein coupled to a scFv targeting CD3 (Dao et al., 2015), inhibited xenograft mouse models of human leukemias and solid cancers.

A slightly different approach is the use of bsAb armed T cells (Lum et al., 2015). An example is HER2Bi, a bsAb consisting of two linked antibodies targeting HER2 and CD3. In a phase 1 study, T cells were harvested from the patient and cultured together with the bsAb. The T cells plus the bsAb were then re-infused (Lum et al., 2015). Due to the

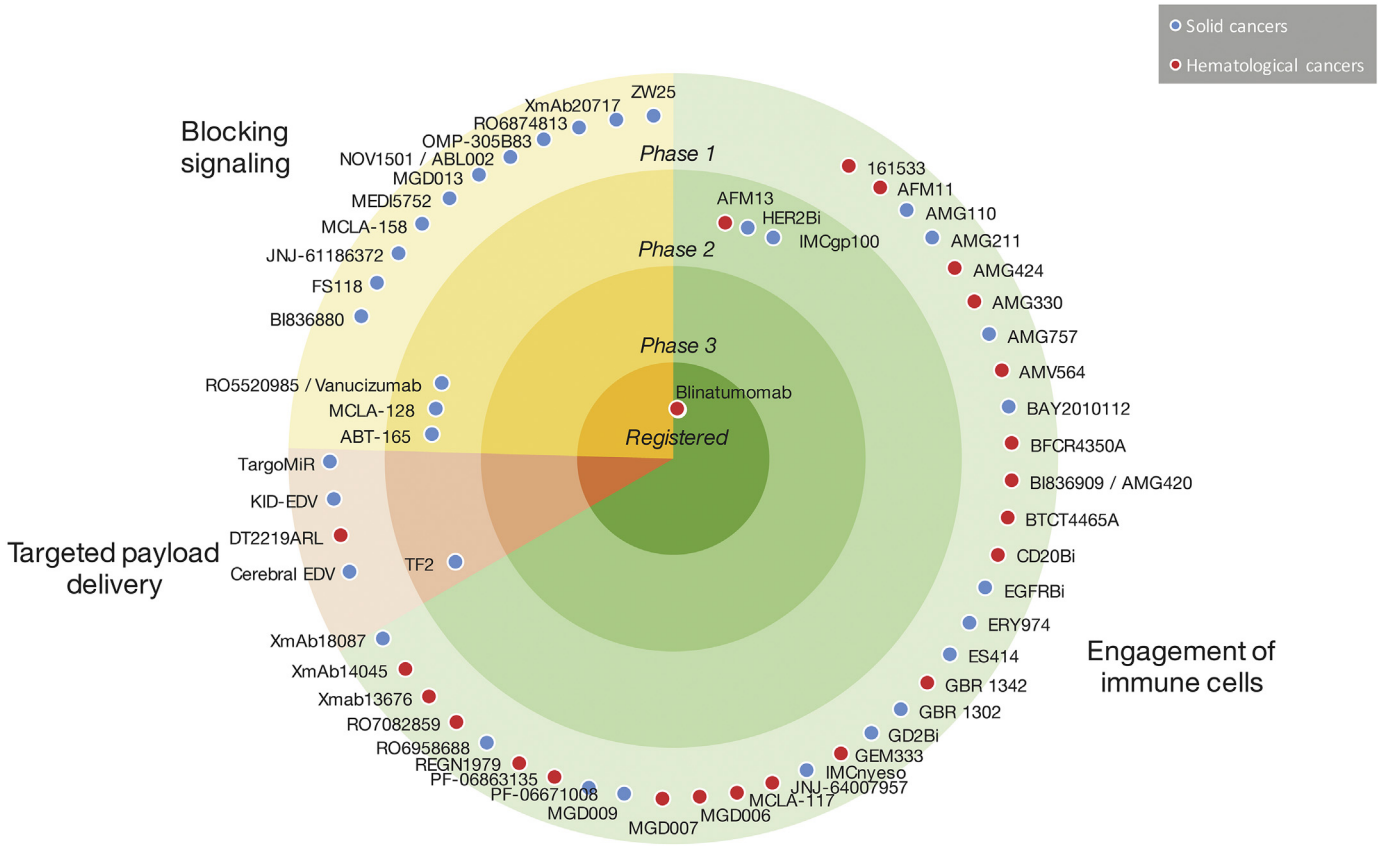

Fig. 3. BsAbs in development and registered in clinical trials at ClinicalTrials.gov in cancer patients. BsAbs are displayed as dots and their location in the chart indicates the most advanced phase of development and their mechanism of action. Registered bsAbs are all shown at the center of the chart and bsAbs in phase 1 are shown at the periphery. The bsAbs are also sorted according to mechanism of action: the green part represents the engagement of immune cells, the red part represents targeted bsAbs and the yellow part represents signal blockade. The color of the dot indicates whether the bsAb is targeted against a solid or hematological cancer.

controlled binding to the T cells ex vivo, less bsAb is potentially required and chance of side effects might be reduced (Bhutani & Lum, 2015). This phase 1 study confirms relatively mild side effects, and showed increased levels of cytokines generally involved in anti-tumor immune responses (Table 2).

4.2. Interplay of CD3+ T cell-engaging bsAbs with the immune system

In general, T cell engaging bsAbs destroy their target independent of co-stimulation, as shown in in vitro cytotoxicity assays with human PBMCs inducing cell death in a human lymphoma cell line in the presence of an anti-CD3 × anti-CD19 bsAb (Dreier et al., 2002). However, addition of a co-stimulatory signal, in this case interleukin-2, can enhance the potency, especially when the PBMCs are co-cultured with the co-stimulatory signal (Dreier et al., 2002). Likewise, targeting co-stimulatory molecules CD137 and CD28 as a co-treatment improved tumor cell killing of immune engaging bsAbs (Liu et al., 2010). Combining a bsAb binding anti-CD137 and anti-CD20 with a bsAb binding anti-CD3 and anti-CD20, showed a synergistic effect in mice bearing human lymphoma xenografts (Liu et al., 2010). However, the CD137 × CD3 bsAb alone did not reduce tumor growth.

Besides co-stimulatory molecules, co-inhibitory molecules are also thought to hamper the effect of immune cell-engaging bsAbs. BsAb RO6958688, the 2:1 CrossMab construct targeting CEA and CD3, increased T cell infiltration into a xenograft colon carcinoma in mice co-grafted with PBMCs as shown with intravital microscopy (Bacac et al., 2016). Moreover administration of this bsAb converted a PD-L1 negative tumor in a PD-L1 positive tumor (Bacac et al., 2016). Similar results were reported for transgenic mouse models with human CD3 and lung and liver carcinoma transduced with human glypican-3 when treated with ERY974, an IgG format bsAb targeting glypican-3 and CD3 (Ishiguro et al., 2017). In in vitro co-cultures of T cells and a panel of tumor cell lines, a BiTE targeting CD3 and CEA induced PD1 expression on T cells and PD-L1 expression on the tumor cells regardless of their initial expression levels (Osada et al., 2015). Cytotoxicity of this BiTE was enhanced by addition of anti-PD1 and anti-PD-L1 antibodies.

HEK293 tumor cells transfected with PD-L1 limited cytotoxic activity in vitro of HER2-TBD, an anti-HER2 x anti-CD3 bsAb (Junttila et al., 2014). In that study, administration of this bsAb combined with a PD-L1 blocking antibody restored the cytotoxic potential of the bsAb (Junttila et al., 2014). Next, in a syngeneic tumor model in transgenic mice expressing human CD3, human HER2-transfected CT26 tumors were treated with the same anti-HER2 x anti-CD3 bsAb alone or in combination with an anti-PD-L1 antibody (Junttila et al., 2014). The combination treatment also controlled the tumor growth more potently (Junttila et al., 2014). An Fab(2)-scFv construct engaging CD3 to TROP-2 was synergistic when combined with an anti-PD1 antibody to inhibit tumor growth in spheroid models of the MDA-MB-231 breast cancer cell line and when xenografted in mice (Chang et al., 2017).

The potential of immune cell engaging bsAbs to increase T cell infiltration into solid tumors (Ji Li et al., 2018) and the emerging evidence that inhibition of the PD1/PD-L1 axis could potentiate the effect of bsAbs, is leading to an increase in phase 1 trials evaluating immune cell engaging bsAbs in combination with checkpoint inhibitors, especially anti-PD-L1 antibodies (Table 3). Early results show enhanced activity of RO6958688, the CEA and CD3 targeting bsAb, when combined with anti-PDL1 antibody atezolizumab in patients with metastatic colorectal cancer (Argilés et al., 2017; Segal et al., 2017). Two of 31 patients treated with RO6958688 alone had a partial response, compared to three of 14 patients treated with the combination (Argilés et al.,

2017; Segal et al., 2017) (Table 2). Moreover, no additive toxicities were seen.

4.3. Engagement of other immune receptors

Besides T cells, other effector cells or immune cell subsets can also be engaged to tumor cells (Lameris et al., 2014). There are many CD3 + T cell subtypes and not all contribute to anti-tumor immune responses. Regulatory T cells (Treg) suppress activated T cells. The amount of Tregs in the peripheral blood prior to blinatumomab treatment inversely predicted response in 42 patients with B cell ALL (Duell et al., 2017). In vitro, blinatumomab activated the Tregs which suppressed the cytotoxicity of effector T cells (Duell et al., 2017). Preventing the activation of Tregs is one of the rationales behind the development of a CD8+ T cell and prostate stem cell antigen engaging tandem scFv (Michalk et al., 2014). This bsAb did induce lysis of a human prostate tumor cell line in vitro, but less effectively compared to a CD3+ T cell engaging bsAb when co-cultured with human PBMCs and isolated CD8+ T cells (Michalk et al., 2014).

A bsAb engaging the agonistic T cell receptor CD28 with CD20 showed robust tumor cell killing in vitro of several lymphoma cell lines co-cultured with PBMCs (Otz, Große-Hovest, Hofmann, Rammensee, & Jung, 2009). The BiTE-like construct RM28 targets CD28 and the TAA melanoma-associated proteoglycan on melanoma cells (Grosse-Hovest et al., 2003). A phase 1 trial in which this bsAb was administered intralesionally in patients with metastatic melanoma was completed in 2007 (NCT00204594), but results are not available.

BsAbs are also developed to target natural killer (NK)s, which are potent cytotoxic lymphocytes of the innate immune system. A phase 1 trial in patients with Hodgkin's lymphoma of AFM13, a tandem diabody (TandAb) construct targeting CD30 and CD16, has been completed (Rothe et al., 2015). In that study, activated NK cells and a decrease of soluble CD30 were seen in the peripheral blood, and three out of 26 patients had a partial remission (Rothe et al., 2015) (Table 2). A phase 2 trial with AFM13 is now ongoing in patients with Hodgkin's lymphoma (Table S1).

A CD16 and CD33 NK-cell engaging bsAb was modified by introducing IL-15 between the anti-CD33 and anti-CD16 blocks (Fig. 1C) (Vallera et al., 2016). It showed superior anti-tumor activity and enhanced survival of human NK cells in vitro compared to the non-modified bsAb (Vallera et al., 2016). A trial of this trispecific construct, known as 161,533, is planned in patients with CD33+ myeloid malignancies (Table S1).

5. Payload delivery

BsAbs are also options for payload delivery. Payload delivery via antibodies, such as radioimmunotherapy and antibody-drug-conjugates, has entered the clinic (Moek, de Groot, de Vries, & Fehrmann, 2017). In this approach, a payload containing an isotope or a drug is directly coupled to an antibody. The radioimmunotherapy ^{90}Y-ibritumomab tiuxetan is registered for the treatment of non-Hodgkin lymphoma, the antibody-drug-conjugate ado-trastuzumab emtansine is registered for the treatment of patients with metastatic HER2 overexpressing breast cancer, and brentuximab vedotin is registered for the treatment of Hodgkin lymphoma and systemic anaplastic large cell lymphoma. They deliver their payload directly to the tumor by binding of the antibody to the TAA. The antibody, with payload, bound to the TAA is then internalized and the payload is trapped in the cell and can exert its effect.

Using a bsAb enables new targeting methods. Instead of direct coupling to an antibody, a bsAb with affinity for the TAA and the payload can be incubated with the payload before injection. Pretargeted delivery could also be achieved by first injecting the bsAb with affinity for a TAA and for a payload, and then injecting the payload. Pretargeting techniques to deliver payloads to a tumor could potentially circumvent prolonged exposure of healthy tissue to the payload, thus mitigating toxicity and adverse effects (Boerman, van Schaijk, Oyen, & Corstens, 2003).

Connecting the payload and the bsAb is achieved by directing one arm of the bsAb to a hapten of the payload (Goldenberg et al., 2012; Goldenberg & Sharkey, 2007; Knight & Cornelissen, 2014). Haptens are molecules that are not immunogenic by themselves, but can act as an antigen and can be bound by an antibody.

The first paper reporting a clinical trial using a bsAb for delivery of a payload was published in 1993 (Le Doussal et al., 1993). Currently, five bsAbs delivering payloads are in clinical trials, four of which target solid tumors. BsAb TF2, existing of three Fab fragments of which two target CEA and one the payload, is most advanced with a phase 2 trial (Fig. 3).

5.1. Pretargeted delivery of a radioactive payload

Patients with medullary thyroid cancer expressing CEA were injected with bsAb TF2, targeting CEA and the payload (Schoffelen et al., 2013). After 24 h, the payload, a small peptide labeled with ^{111}indium, was administered. Tumor-to-tissue ratios >1:20 were observed 24 h after administering this small peptide showing the feasibility of pretargeting with bsAbs (Schoffelen et al., 2013). In theory, the unbound payload will be cleared rapidly due to its small size, minimizing damage to not-targeted tissues (van de Watering, Rijpkema, Robillard, Oyen, & Boerman, 2014).

When the payload is a therapeutic radiometal, the hapten can be the chelator of the radiometal (Cheal et al., 2014). Another option is the use of two haptens to create one large bivalent hapten that favors the binding to two tumor-bound bsAbs, which would stabilize binding to the tumor (Barbet et al., 1999). This system is called affinity enhancement system (Le Doussal, Martin, Gautherot, Delaage, & Barbet, 1989) and has been used in clinical studies (Table 2).

For the pretargeted delivery of yttrium-90 for radioimmunotherapy, a bsAb with affinity for CD38 and the DOTA-yttrium complex was compared with an antibody binding the radiometal via a streptavidin-biotin bond. In mice xenografted with non-Hodgkin lymphoma, or multiple myeloma, the bsAb approach showed a superior antitumor effect compared to the streptavidin-biotin approach (Green et al., 2018).

Pretargeting can also be achieved with alternatives for linking the payload and the antibody. These include streptavidin-biotin, oligonucleotides or click-chemistry, such as the cycloaddition reaction between a tetrazine and a trans-cyclooctene (Altai, Membreno, Cook, Tolmachev, & Zeglis, 2017). However the approach with bsAbs is the only one that has been tested in the clinic so far (Altai et al., 2017) (Table 2).

5.2. Delivery of other payloads

Pretargeted delivery of other toxic payloads by bsAbs, such as doxorubicin, has been explored in animal models by binding a chelator-hapten (Gada, Patil, Panwar, Hatefi, & Khaw, 2012; Khaw et al., 2014). In these studies, the chelator was loaded with the radioisotope technetium-99 to validate target-specific binding. Other haptens, such as digoxigenin, can also be conjugated to the payload and are used for drug delivery (Dengl, Sustmann, & Brinkmann, 2016). Several payloads, such as doxorubicin and the fluorescent dye Cy5 conjugated to digoxigenin, showed specific targeting in human xenograft mouse models (Metz et al., 2011).

A direct targeting approach, in which the bsAb and the payload are incubated prior to administration is being tested in the clinic (MacDiarmid et al., 2007) (Table 2 and S1). In this approach, the payload is encapsulated in a bacterially-derived nanocell, which is called an engeneic delivery vehicle (EDV), and the bsAbs are two antibodies linked together via their Fc regions (MacDiarmid et al., 2007). The payload can be a chemotherapeutic drug such as doxorubicin or paclitaxel, but also silencing microRNA. Results of three trials that tested EDVs

Table 1
Constructs of the bsAbs in clinical trials.

Construct	Structure	Characteristics	bsAbs
TrioMab	Produced in a rat/mouse quadroma (Chelius et al., 2010). One heavy-light chain is rat, the other heavy-light chain is mouse.	Species restricted heavy-light chain pairing	Catumaxomab
IgG-like, common light chain.	IgG like with each Fab binding another epitope.	Heterodimerization of heavy chains is based on the knob-in-holes or a another heavy chain pairing technique. Randomly pairs light chains to heavy pairs. Often a common light chain is used (Dovedi et al., 2018), (E. J. Smith et al., 2015), (Yen et al., 2016), (de Vries Schultink et al., 2018).	ERY972, BTCT4465A, MCLA-117, MCLA-128, MEDI5752, OMP305B83, REGN1979, ZW25
CrossMab	Uses the knob-in-holes technique for the heavy chain pairing. The C_H1 domain of the heavy chain is switched with the constant domain of the light chain (C_L) (Klein, Schaefer, & Regula, 2016).	Ensures specific pairing between the heavy-light chains. No side products possible.	Vanucizumab
2:1 CrossMab	An additional Fab-fragment is added to the N-terminus of its VH domain of the CrossMab (Klein et al., 2016), (Bacac et al., 2018).	The added Fab-fragment to the CrossMab increases the avidity by enabling bivalent binding.	RO6958688, RO7082859
2:2 CrossMab	A tetravalent bispecific antibody generated by fusing a Fab-fragment to each C-terminus of a CrossMab (Klein et al., 2016). These Fab-fragments are crossed: their CH1 is switched with their CL. VH is fused to their CL and the VL to the CH1 (Brünker et al., 2016).	CrossMab technology in Fab-fragments ensure specific pairing. Avidity is enhanced by double bivalent binding.	RO6874813
Duobody	The Fab-exchange mechanism naturally occurring in IgG4 antibodies is mimicked in a controlled matter in IgG1 antibodies, a mechanism called controlled Fab exchange (Labrijn et al., 2013).	Ensures specific pairing between heavy-light chains and heterodimerization of heavy chains.	JNJ-61186372, JNJ-64007957
Dual-variable-domain antibody (DVD-Ig)	Additional V_H and variable light chain (V_L) domain are added to each N-terminus for bispecific targeting (Jakob et al., 2013).	This format resembles the IgG-scFv, but the added binding domains are bound individually to their respective N-termini instead of a scFv to each heavy chain N-terminus.	ABT165
scFv-IgG	Two scFv are connected to the C-terminus of the heavy chain (C_H3) (Xu et al., 2013).	Has two different bivalent binding sites and is consequently also called tetravalent. No heavy-chain and light-chain pairing problem.	MM-141, NOV1501/ABL001
IgG-IgG	Two intact IgG antibodies are conjugated by chemically linking the C-terminals of the heavy chains (Ma et al., 2013).	Facile development using available antibodies.	EGFRBi, HER2Bi, Cerebral EDV, KIDEDV, TargoMir
Fab-scFv-Fc	Assembly of a light chain, heavy chain and a third chain containing the Fc region and the scFv (Moretti et al., 2013), (Chu et al., 2014), (de Zafra et al., 2017).	Efficient manufacturing and purification.	XmAb14045, XmAb13676, XmAb18087, XmAb20717, AMG424, GBR1302, GBR1342
TF	Three Fab fragments are linked by disulfide bridges (Rossi et al., 2006). Two fragments target the tumor associated antigen (TAA) and one fragment targets a hapten.	Lacks an Fc region.	TF2
ADAPTIR	Two scFvs bound to each sides of an Fc region (Hernandez-Hoyos et al., 2016).	Abandons the intact IgG as a basis for its construct, but conserves the Fc region to extend the half-life and facilitate purification.	ES414
Bispecific T cell Engager (BiTE)	Consists of two scFvs, V_LA V_HA and V_HB V_LB on one peptide chain (Mack et al., 1995).	Has only binding domains, no Fc region.	Blinatumomab, AMG110, AMG211, AMG330, BAY2010112, BFCR4350A and BI836909/AMG420
BiTE-Fc	An Fc region is fused to the BiTE construct (Lorenczewski et al., 2017).	Addition of Fc region enhances half-life leading to longer effective concentrations, avoiding continuous IV (Arvedson et al., 2017).	AMG757
Dual affinity retargeting (DART)	Two peptide chains connecting the opposite fragments, thus V_LA with V_HB and V_LB with V_HA, and a sulfur bond at their C-termini fusing them together (Moore et al., 2011).	Sulfur bond supposed to improve stability over BiTEs.	MGD006
DART-Fc	An Fc region is attached to the DART structure. Generated by assembling three chains. Two via a disulfide bond, as with the DART. One chain contains half of the Fc region which will dimerize with the third chain, only expressing the Fc region (Moore et al., 2018), (Root et al., 2016).	Addition of Fc region enhances half-life leading to longer effective concentrations, avoiding continuous IV.	MGD007, MGD009, PF-06671008
Tetravalent DART	Four peptide chains are assembled. Basically, two DART molecules are created with half an Fc region and will dimerize (La Motte-Mohs et al., 2017).	Bivalent binding to both targets, thus a tetravalent molecule	MGD013
Tandem diabody (TandAb)	Two diabodies. Each diabody consists of an V_HA and V_LB fragment and a V_HA and V_LB fragment covalently associating. Two diabodies are linked with a peptide chain (Kipriyanov et al., 1999).	Designed to improve stability over the diabody consisting of two scFvs (Kipriyanov et al., 1999). Has two bivalent binding sites.	AFM11, AFM13, AMV564
scFv-scFv-toxin	Toxin and two scFv with a stabilizing linker (Vallera, Chen, Sicheneder, Panoskaltsis-Mortari, & Taras, 2009).	Specific delivery of payload	DT2219ARL
Modular scFv-scFv-scFv	One scFv directed against the TAA is tagged with a short recognizable peptide is assembled to a bsAb consisting of two scFvs, one directed against CD3 and one against the recognizable peptide (Arndt et al., 2014).	Modular system, thus flexible, built around the recognizable peptide.	GEM333

(continued on next page)

Table 1 (*continued*)

Construct	Structure	Characteristics	bsAbs
ImmTAC	A stabilized and soluble T cell receptor is fused to a scFv recognizing CD3 (Oates, Hassan, & Jakobsen, 2015).	By using a TCR, the ImmTAC is suitable to target processed, e.g. intracellular, proteins.	IMCgp100, IMCnyeso
Tri-specific nanobody	Two single variable domains (nanobodies) with an additional module for half-life extension (I. Hofmann et al., 2015).	Extra module added to enhance half-life.	BI836880
Trispecific Killer Engager (TriKE)	Two scFvs connected via polypeptide linkers incorporating human IL-15 (Vallera et al., 2016).	Linker to IL-15 added to increase survival and proliferation of NKs	161,533

have been published (Table 2). The phase 1 data showed an acceptable safety profile.

The bsAb DT2219 has a directly conjugated payload and targets both CD22 and CD19 to enhance specific delivery. The payload is the toxin diphtheria and enters the cytosol after internalization by CD19 and/or CD22 (Bachanova et al., 2015). This bsAb has been studied in patients with refractory B cell malignancies and one complete and one partial response were reported out of 25 patients (Table 2).

6. Signaling blockade

Targeting multiple epitopes or receptors in cancer with combination therapies is a popular approach and many combinational approaches to antibody treatments are being evaluated in clinical trials (D. S. Chen & Mellman, 2017; Henricks, Schellens, Huitema, & Beijnen, 2015; Smyth, Ngiow, Ribas, & Teng, 2016).

A combination of nivolumab, an anti-PD-1 antibody, with ipilimumab, an anti-CTLA4 antibody, has been approved by the FDA and EMA for metastatic melanoma (Postow et al., 2015). Recently, this combination was also approved for the treatment of advanced renal cell carcinoma by the FDA (Motzer et al., 2018). A slightly different combination treatment is a multi-epitope approach with pertuzumab and trastuzumab, both targeting HER2 but on different epitopes. It has been approved as a combination treatment for patients with metastatic HER2-positive tumors (Swain et al., 2015).

Theoretically, the targets of two antibodies could be incorporated into a single bsAb, which could yield various benefits. The specificity of such a drug might be enhanced by co-localization of receptors on cancers, thus minimizing on-target toxicity of healthy tissues. Also, improvements of binding affinity might be achieved by targeting different epitopes of one antigen. Potential disadvantages of such a bsAb are that it would limit itself to one combination of antigens, while antibodies can be combined freely, and it would prevent the sequential administration or personalized dosing of two antibodies. According to ClinicalTrials.gov, 14 bsAbs that block signaling important for the tumor are being studied in clinical trials.

6.1. Tumor cell surface receptors

Due to their crosstalk, common targets for bsAbs that disrupt two signals are the ErbB family members, EGFR, HER2 and HER3 (Fitzgerald et al., 2014; Huang et al., 2013; McDonagh et al., 2012; Moores et al., 2016; Weidle, Kontermann, & Brinkmann, 2014).

BsAbs MM-111, JNJ-61186372 and MEHD7945A are examples that are directed against one or more of these targets (Table S1). They do so with different constructs, although all have a long half-life (Table 1).

Interestingly, bsAb MEHD7945A, targeting EGFR and HER3, is more effective than either the anti-EGFR antibody cetuximab or the EGFR kinase inhibitor erlotinib and overcomes cetuximab or erlotinib resistance in mice xenografted with human non-small cell lung cancer and head and neck squamous cell carcinoma. Most likely this is due to shutting down crosstalk in the signaling pathways of the ErbB family members (Huang et al., 2013). Nevertheless, no benefit of MEHD7945A over cetuximab was found in phase 2 trails in patients with metastatic colorectal cancer (Hill et al., 2018) and head and neck squamous cell carcinoma (Fayette et al., 2016). Therefore development of this bsAb has stopped (Table 2).

Other targets that are being investigated are death receptors, such as CD95, or receptors involved in lysosomal internalization, such as CD63. A bsAb targeting CD20 and CD95, was more effective in inhibiting tumor growth in human xenograft mouse models than different anti-CD20 antibody variants (Nalivaiko et al., 2016). To improve antibody drug conjugates, a bsAb loaded with a drug was designed that bound the receptor CD63 in addition to HER2. This induced internalization, as shown with fluorescent confocal microscopy, and improved tumor inhibition of HER2-positive xenograft mouse models (de Goeij et al., 2016).

The CD47-SIRPα interaction, also called the "don't eat me signal", inhibits phagocytosis of CD47-expressing cells via SIRPα expressed on macrophages (Jaiswal et al., 2009) and is overexpressed on many solid and hematological tumor cells (Willingham et al., 2012). This interaction can also be disrupted by bsAbs. In mice xenografted with Raji tumor cells, an IgG-scFv bsAb targeting CD20 and CD47 prolonged survival and an IgG-like bsAb targeting CD19 and CD47 eradicated the tumor (Dheilly et al., 2017; Piccione et al., 2015), while monotherapies with anti-CD47, anti-CD20 or anti-CD19 antibodies were not effective.

Targeting SIRPα did not induce tumor regression in mice xenografted with Burkitt's lymphoma (Ring et al., 2017), although combination with the anti-CD20 antibody rituximab resulted in synergistic effects, and a bsAb targeting SIRPα and CD70 slowed tumor growth. However, the bsAb yielded the same reduction in tumor growth as an anti-SIRPα antibody combined with an anti-CD70 antibody.

6.2. Immune receptors

Following the establishment of immune checkpoint inhibitors and combinations thereof as therapies in oncology, bsAbs are being explored as additions or improvements to these existing therapies. Tetravalent dual affinity retargeting (DART) construct MGD013 targets both lymphocyte activation gene 3 (LAG-3) and PD-1 bivalently; it will be evaluated in a clinical trial in patients with advanced solid tumor (LaMotte-Mohs et al., 2016). In vitro, MGD013 gave rise to increased cytokine release by T cells compared to monotherapies or combination therapies, indicating increased T cell activation (LaMotte-Mohs et al., 2016).

MEDI5752 is a monovalent antibody combining PD-1 and CTLA-4 inhibition preferentially on tumor-infiltrated lymphocytes (Dovedi et al., 2018). This will be tested in a clinical trial in patients with advanced solid tumors (Table S1).

IgG-like construct FS118 also blocks two pathways by targeting PD-L1 via its Fab-fragments and LAG-3 via its Fc region (Kraman et al., 2017). A murine counterpart of FS118, targeting murine LAG-3 and PD-L1, induced dose-dependent anti-tumor activity (Kraman et al., 2017) and changed the composition of immune infiltrating lymphocytes by increasing the ratio CD8:Tregs (Kraman et al., 2018). This construct is being tested in a clinical trial in patients with advanced cancer (Table S1).

6.3. Inhibiting angiogenesis

Instead of binding two cell membrane epitopes, the tumor environment itself can also be a target. The CrossMab construct vanucizumab

Table 2
Clinical results of bsAbs.

bsAb	Phase	Indication	Dose	Key results	Ref
Blinatumomab (CD19 x CD3)	III	Adults with heavily pretreated B cell precursor ALL (n = 376)	9 μg/d cIV over 1 week, followed by 28 μg/d cIV for 3 weeks	Blinatumomab treated: OS: 7.7 months, CR:44%, grade 3+ AE: 87%. Chemotherapy treated: OS: 4.0 months, CR:25%, grade 3+ AE: 92%.	(Kantarjian et al., 2017)
Blinatumomab (CD19 x CD3) + tyrosine kinase inhibitor	Retrospective	Adults with relapsed/refractory Ph + acute lymphoblastic leukemia (n = 9) and chronic myeloid leukemia in blast crisis (n = 3)	blinatumomab and a TKI (ponatinib, n = 8; dasatinib, n = 3; bosutinib, n = 1)	OS: not reached after 14 months, CR: 9/12, AE: 2/12 grade 2 cytokine release syndrome.	(Assi et al., 2017)
Catumaxomab (EpCAM x CD3)	II/III	Malignant ascites secondary to epithelial cancers (n = 258)	10, 20, 50 and 150 μg/day on day 0, 3, 7, 10, respectively, via IP infusion	Catumaxomab plus paracentesis treated: OS:72 days, puncture free survival: 46 days. Paracentesis treated: OS: 68 days, puncture free survival: 11 days. AE: 23% of patients had a serious adverse event. AE: pyrexia (60.5%), abdominal pain (42.7%), nausea (33.1%), vomiting (27.4%).	(Heiss et al., 2010)
MEHD7945A/Duligotuzumab (EGFR x HER3)	II	RAS wild-type metastatic colorectal cancer (n = 134)	Duligotuzumab 1100 mg IV every 2 weeks + FOLFIRI (n = 68) Cetuximab 400 mg/m^2 iv, followed by 250 mg/m^2 IV weekly + FOLFIRI (n = 66)	Patient outcomes not improved, development stopped. PFS: 7.3 vs 5.7 months, OS: 14 vs 12.4 months, CR: 0% vs 3%. Duligotuzumab vs cetuximab, respectively. AE: rash (84%), diarrhea (79%), fatigue (62%), and nausea (50%). Similar G ≥ 3 AEs between treatment groups.	(Hill et al., 2018)
MEHD7945A/Duligotuzumab (EGFR x HER3)	II	Head and neck squamous cell carcinoma (n = 121)	Duligotuzumab: 1100 mg iv every 2 weeks (n = 59) Cetuximab: 400 mg/m^2 iv, followed by 250 mg/m^2 iv weekly (n = 62)	PFS: 4.2 vs 4.0 months, OS: 7.2 vs 8.7 months, CR: 2% vs 18%. Duligotuzumab vs cetuximab, respectively. AE: rash, infections diarrhea, fatigue, and nausea. G ≥ 3 AEs in the duligotuzumab arm (61%) versus cetuximab arm (51%)	(Fayette et al., 2016)
AFM13 (CD30 x CD16A)	I	Relapsed or refractory Hodgkin's lymphoma (n = 28)	Weekly infusion for 4 weeks. 0.01, 0.04, 0.15, 0.5, 1.5, 4.5, and 7.0 mg/kg body weight	PR: 11.5%, SD: 50%. AE: fever (53.6%), chills (39.3%), headache (28.6%), nausea and nasopharyngitis (17.9%), and infusion reaction, rash, vomiting, and pneumonia (14.3%). MTD not reached.	(Rothe et al., 2015)
AMG110 (EpCAM x CD3)	I	Relapsed or refractory solid tumors (n = 65)	1–96 μ/day clVfor ≥28 days	MTD: 24 μ/day. SD: 18/64. AE: Diarrhea (46%), pyrexia (43%), peripheral edema (40%), nausea (39%), vomiting (34%), abdominal pain (32%), AE ≥ G: 95%.	(Kebenko et al., 2018)
AMG211 (CEA x CD3)	I	Relapsed of refractory gastrointestinal adenocarcinoma (n = 44)	0.2–12.8 μg/day clV for 1–3 weeks.	Disease progression in 33/44 pts. AE: fatigue, nausea, abdominal pain, pyrexia and diarrhea.	(Moek et al., 2018)
BI836880 (VEGF x Ang-2)	I	Advanced or metastatic solid cancer (n = 29)	Schedule 1: 40–1000 mg every three weeks. Schedule 2:40–180 mg every week.	MTD/RP2D: 720 mg every three weeks. PR: 7%, SD: 31%. AE: Hypertension (86%), asthenia (48%), nausea (45%) and vomiting (38%).	(Le Tourneau, Claus, et al., 2018; Le Tourneau, Tabernero, et al., 2018)
BIS-1 (EpCAM x CD3)	I	Malignant peritoneal or pleural effusion (n = 9)	Autologous activated T lymphocytes in the presence of BIS-1 were locally infused in patients with peritoneal or pleural effusion.	Effusions showed a reduction or complete disappearance of tumor cells 24 h after start of treatment.	(Kroesen et al., 1997)
		Renal cell cancer (RCC) (n = 14)	RCC patients received 1, 3 or 5 μg/kg BIS-1 (without Fc region) accompanied with SC interleukin-2 therapy.	For BIS-1 without Fc region, no responses were seen. AE: Severe toxicity observed at 3 and 5 μg/kg	

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Table 2 (*continued*)

bsAb	Phase	Indication	Dose	Key results	Ref
CD20Bi (CD20 x CD3)	I	Lymphoma and myeloma (n = 12)	5, 10, 15, 20 or 40 × 10^9 T cells incubated with CD20Bi per infusion	AE: chills, fever, hypotension, fatigue. MTD not reached.	(Lum et al., 2013)
DT2219 (CD19 x CD22)	I	Refractory B cell malignancies (n = 25)	0.5, 1.25, 2.5, 5, 10, 20, 40, 60, 80 μg/kg/day every other day for 4 total doses (days 1, 3, 5, and 8)	CR:1/25, PR: 1/25 RP2D: 40–80 μg/kg/day. AE: weight gain (range, 5%–14% of baseline), peripheral edema, and hypoalbuminemia consistent with capillary leak syndrome, grade 1–2 fever, and fatigue.	(Bachanova et al., 2015)
EGFRBi (EGFR x CD3)	I	Advanced pancreatic and colon cancer (n = 5)	10, 20 or 40 × 10^9 T cells incubated with EGFRBi by infusion	OS: 14.5 months, AE: grade 1–2 headaches, fevers, chills and blood pressure changes. MTD not reached.	(Lum, 2015)
EGFR-nanocell-paclitaxel	I	Advanced solid tumors (n = 28)	1 × 10^8, 1 × 10^9, 3 × 10^9, 1, 1.5, 2, 5 × 10^{10} nanocells per weekly infusion, 5 weeks	SD:10/22 patients, MTD: 1 × 10^{10}, RP2D: 5 × 10^9 nanocells per infusion. AE: grade 4 lymphopenia (2/28), grade 4 elevated aminotransferase (1/28) and grade 4 elevated alanine transaminase (1/28). Common AE: transient chills (16/28, 57%) and pyrexia (13/28, 46%).	(Solomon et al., 2015)
EGFR-nanocell-doxorubicin	I/II	Recurrent glioblastoma (n = 16)	1 × 10^9, 2 × 10^9, 5 × 10^9, and 8 × 10^9 nanocells per weekly infusion, 8 weeks	OS: 9 months and 21 days, SD: 28%, RP2D: 8 × 10^9 nanocells per infusion. No DLT observed. AE: nausea (7/16), fever (5/16), and chills or rigors (5/16).	(Whittle et al., 2015)
F6–734/hMN14–734 (CEA x DTPA)	Retrospective	Metastatic medullary thyroid cancer (n = 29) versus control metastatic medullary thyroid cancer (n = 39)	20–50 mg of anti-CEA/anti–DTPA-indium murine BsMAb F6–734, 4 days later the hapten labeled with 1.4 to 4.1 GBq of ^{131}iodine. Or 40 or 75 mg/m^2 humanized anti-CEA/murine anti–DTPA-indium BsMAb (hMN14–734), 5 days later 2.7 GBq of ^{131}iodine labeled hapten.	100% increase in serum calcitonin doubling times (defined as biologic responder) and bone-marrow involvement are prognostic indicators in patients. OS biologic responders: 159 months, OS non-responders: 109 months, OS untreated: 61 months. AE: grade 4 thrombocytopenia (5/29) and grade 4 neutropenia (4/29)	(Chatal et al., 2006)
FBTA05 (CD20 x CD3)	I	recurrent or refractory B cell malignanciesRecurrent or refractory B cell malignanciess Pediatric recurrent or refractory B cell malignancies (n = 10)	Individual treatment schedules. Doses from 10 to 300 μg weekly or 10–100 μg daily.	CR: 5/10, PR: 1/10, SD: 3/10. AE: acute infusion reactions, fatigue, hypotension.	(Schuster et al., 2015)
HER2Bi (HER2 x CD3)	I	Metastatic breast cancer (n = 22)	5, 10, 20 or 40 × 10^9 T cells incubated with HER2Bi per infusion	SD: 13/22, PD: 9/22, OS HER2 3+ patients: 36.2 months, OS HER2 0–2+: 27.4 months. AE: grade 3 chills and grade 3 headaches. Nausea/diarrhea: 9/22 patients. MTD not reached.	(Lum et al., 2015)
IMCgp100 (gp100 x CD3)	I	Metastatic uveal melanoma (n = 19)	Week 1: 20 μg iv, once. Week 2: 30 μg iv, once. Week 3 and beyond: 60, 70, 80, 75 μg per week	MTD/R2PD: 75 μg. SD:12/19. AE: pruritus (84%), pyrexia (84%), fatigue (74%), hypotension (15%), peripheral edema (63%).	(Sato et al., 2017)
IMCgp100 (gp100 x CD3)	I	Advanced melanoma (n = 31)	5 ng/kg to 900 ng/kg IV every week or daily	MTD: 600 ng/kg weekly iv PR: 4/26, SD: 12/26, AE: rash (100%), pruritus (64%), pyrexia (50%), and periorbital edema (46%).	(Middleton et al., 2016)
LY3164530 (MET x EGFR)	I	Advanced or metastatic cancer (n = 29)	Schedule 1:300–1250 mg every 2 weeks. Schedule 2: 500–600 mg weekly.	Development stopped due to toxicity and lack of potential predictive biomarker. MTD schedule 1:1000 mg MTD schedule 2: 500 mg OR: 10.3%, SD: 17.2%. AE: Acneiform (84%), hypomagnesemia (55.2%), paronychia (34.5%).	(Patnaik et al., 2018)
MCLA-128 (HER2 x HER3)	I/II	Advanced solid tumors (n = 28)	40–900 mg every 3 weeks IV over 1–2 h Phase 2 part, at RP2D	No DLT observed. RP2D: 750 mg every 3 weeks. Phase 2: 8 patients with HER2 amplified metastatic breast cancer. PR:1/8, SD: 5/8, AE: infusion related effects (40%), G1–2	(Alsina et al., 2017)

Table 2 (*continued*)

bsAb	Phase	Indication	Dose	Key results	Ref
				diarrhea (13%), rash (13%), fatigue (13%).	
MDX-447 (EGFR x CD64)	I	Advanced solid tumors (n = 64)	1–40 mg/m^2, IV weekly. 1 to 15 mg/m^2 in combination with G-CSF (3 μg/kg) sc	MTD MDX-447 alone: 30 mg/m^2 CR: 0, PR: 0 AE: 633 administrations, 41 grade 3 or 4 event containing: hypotension (7), dyspnea (5), pain (3), hypertension (3), headache (2), fever (2), diarrhea (2), thrombocytopenia (2), and hyperglycemia (2).	(Fury, Lipton, Smith, Winston, & Pfister, 2008)
MM-111 (HER2 x HER3)	I	HER2+ cancers (n = 86)	10, 20, 30 and 40 mg/kg, weekly	MTD not reached. RP2D: 20 mg/kg weekly and 40 mg every 3 weeks. CR:1/74, PR: 18/74, SD: 26/74.	(Richards et al., 2014)
MM-141 (IGF-1R x HER3)	I	Hepatocellular carcinoma, relapsed or refractory solid tumors (n = 42)	Weekly doses of 6, 12 or 20 mg/kg, or biweekly doses of 40 mg/kg. Cohort expansion at 20 mg/kg/week group	Patients with detectable serum levels of free IGF-1 prior to the start of therapy remained on study longer than those with undetectable levels (9 vs 15.7 weeks). SAE in >20% of patients: nausea, vomiting, decreased appetite and headache.	(Isakoff et al., 2016)
OMP-305B83 (DLL4 x VEGF)	I	Previously treated solid cancers (n = 49)	0.5–10 mg/kg every 3 weeks	PR: 1/39, SD: 14/39. AE: systemic hypertension (54%), fatigue (20%), headache (24%), anemia (13%), dyspnea (11%).	(Jimeno et al., 2016)
RG7802, RO6958688 (CEA x CD3)	I	Advanced CEA+ solid tumors (n = 118)	Group 1: 0.05–600 mg, Group 2: combined with 1200 mg atezolizumab (anti-PDL1): 5–160 mg per week	Group 1: PR: 2/31, AE: pyrexia (56%), infusion related reaction (50%), diarrhea (40%). DLTs: G3 dyspnea, G3 diarrhea, G4 colitis and G5 respiratory failure. Group 2: PR: 3/14, no additive toxicities.	(Argilés et al., 2017; Segal et al., 2017; Tabernero et al., 2017)
RO6874813 (FAP x DR5)	I	Advanced solid tumors (n = 32)	0.5–45 mg/kg every week or every other week	MTD: not reached. PR: 1/31, SD: 6/31. AE: fatigue (21.9%), nausea (15.6%), and infusion-related reactions (9.4%). AEs ≥ G3: anemia (3.6%) and asthenia (3.6%)	(Bendell et al., 2018)
TargoMIRs (EGFR x EDV-miR16)	I	Malignant pleural mesothelioma (n = 27)	5×10^9, 7×10^9, and 9×10^9 TargomiRs either once or twice weekly IV. After eight patients, all subsequent patients 1×10^9 TargomiRs.	MTD: 5×10^9 TargomiRs. PR: 1/22, SD: 15/22. AE: transient lymphopenia (25/26), temporal hypophosphatemia (17/26).	(van Zandwijk et al., 2017)
TF2 + IMP288 (CEA x IMP288)	I	CEA+ colorectal cancers (n = 20)	Imaging with ^{111}indium to confirm tumor targeting. If targeting confirmed, then treated with 2.5–7.4 GBq ^{177}lutetium. TF2: 75-150 mg, 1 or 5 days later IMP288: 25–100 μg	Rapid imaging possible, tumor to tissue ratio > 20:1 after 24 h. No tumor responses observed. AE: grade 3/4 thrombocytopenia (1/20), and grade 3 lymphopenia (1/20).	(Schoffelen et al., 2013)
TF2 + IMP288 (CEA x IMP288)	I	Medullary thyroid carcinoma (n = 15)	60-120 nmol TF2, 3–6 nmol IMP288, 24-42 h between injections. Positron emission tomography:1–2 h after injection	Imaging protocol. 30 h between injection and TF2/IMP288 ratio of 20 is optimal.	(Bodet-Milin et al., 2016)
Vanucizumab (Ang-2 x VEGF-A)	I	Cisplatin resistant ovarian cancer (n = 41)	30 mg/kg IV every 2 weeks	PR: 29% (12/41), SD: 53% (21/41). AE: hypertension (53%), asthenia (39%), constipation (34%), abdominal pain (32%), peripheral (24%)/lymphedema (19%), vomiting (24%), diarrhea (19%). AEs ≥ G3: hypertension (10/24%), pyelonephritis (3/7%), GI-perforation, peritonitis, intestinal obstruction, pulmonary embolism, dyspnea (2/5%).	(Oaknin et al., 2015)
ZW25 (HER2 x HER2)	I	HER2+ cancers (n = 9)	5, 10 mg/kg. 15 mg/kg planned	PR: 2/8, SD:1/8. AE: infusion reaction (5/9), diarrhea (4/9), fatigue (3/9).	(Meric-Bernstam et al., 2017)

IV, intravenously; IP, intraperitoneal; SC, subcutaneously; OR, overall response; CR, complete response; PR, partial response; SD, stable disease; OS, overall survival; DLT, dose limiting toxicity; MTD maximum tolerable dose; AE, adverse event; RP2D, recommended phase 2 dose; G1–4, grade 1–4.

inhibits angiogenesis by depleting angiogenin-2 (Ang-2) and vascular endothelial growth factor-A (VEGF-A) in the tumor environment. The bsAb OMP-305B83 targets delta-like ligand 4 and VEGF. In this construct, both bsAbs are Fc-bearing since a long half-life is paramount to effective depletion of factors.

Vanucizumab inhibited tumor growth and metastasis in mice bearing multiple syngeneic, patient-derived and xenograft tumor models (Kienast et al., 2013). It also increased activation of intratumoral immune cells leading to upregulated PD-L1 expression by endothelial cells (again in multiple syngeneic mouse models) (Schmittnaegel et al., 2017). In this approach, adding anti-PD-1 antibody treatment to vanucizumab increased survival providing further rational to evaluate this bsAb in combination with immunotherapies (Table 3).

6.4. Increasing specificity

The bsAb RO6874813, a 2:2 CrossMab, involves a different approach. It has affinity for the death receptor (DR) 5, one of the activating TNF-related apoptosis-inducing ligand receptors on tumor cells, and for fibroblast activation protein (FAP) on cancer-associated fibroblasts. In contrast to previous attempts with antibodies to activate DR5 on tumor cells, this bsAb enhances specificity to the tumor by using the affinity for the cancer-associated fibroblasts (Brünker et al., 2016). In in vitro and in human xenograft mouse models with fibroblasts combined with different carcinomas or a patient-derived sarcoma, the efficacy of this bsAb depended on the presence of cancer-associated fibroblasts. In in vivo models, the bsAb inhibited tumor growth more effectively than the anti-DR5 therapy (Brünker et al., 2016).

7. Remaining challenges

The approval of blinatumomab and emicizumab have stimulated the influx of bsAbs into clinical trials (Fig. 4). Continuous administration of small bsAbs, like blinatumomab, is necessary to maintain a constant blood level when treating patients (Portell et al., 2013). One way to circumvent this drawback is by prolonging the half-life of the bsAbs by adding an Fc region (Arvedson et al., 2017; L. Liu et al., 2017; Lorenczewski et al., 2017).

At present, two popular small bsAb platforms, the BiTE and the DART construct, both have an Fc region extended version in clinical trials (Fig. 1C). AMG757, targeting DLL3 and CD3, is a BiTE-Fc; MGD007 and MGD009, targeting glycoprotein A33 and CD3 and B7-H3 and CD3, respectively, are DART-Fc constructs. All these bsAbs target solid tumors. MGD007 has recently completed a phase 1 clinical trial in patients with relapsed or refractory metastatic colorectal carcinoma (NCT02248805). The results have not been published. However, the study design of the MGD007 illustrates the advantage of a longer half-life; weekly and three-weekly treatment regimens are used, while the DART molecule MGD006, targeting CD123 and CD3, is administered via continuous IV infusion to patients with AML (NCT02152956). An increasing number of novel bsAbs entering clinical trials have an Fc region (Fig. 4).

Moreover, blinatumomab is administered via stepwise dosing to mitigate toxicity (Topp et al., 2015). The severe toxicity of this construct is caused by systemic cytokine release called cytokine release syndrome and is commonly found in T cell-engaging therapies (Maude, Barrett, Teachey, & Grupp, 2014). Besides stepwise dosing, corticosteroids are

Table 3
BsAbs in clinical trials in combination with immune modulators.

bsAb	Immunotherapy	Phase	Indication	NCT number	Status
ABT-165 (DLL4 x VEGF)	ABBV-181 (anti-PD-1 mAb)	I	Advanced solid tumors	NCT01946074	Active, not recruiting
AFM13 (CD30 x CD16A)	Pembrolizumab (anti-PD-1 mAb)	I	Hodgkin lymphoma	NCT02665650	Active, not recruiting
BI836880 (Ang2 x VEGF)	BI754091 (anti-PD-1 mAb)	I	Non-squamous, Non-small-cell lung cancer	NCT03468426	Recruiting
Blinatumomab (CD19 x CD3)	Nivolumab (anti-PD-1 mAb) ipilimumab (anti CTLA4 mAb)	I	B acute lymphoblastic leukemia	NCT02879695	Recruiting
Blinatumomab (CD19 x CD3)	Pembrolizumab (anti-PD-1 mAb)	I	B acute lymphoblastic leukemia	NCT03160079	Recruiting
Blinatumomab (CD19 x CD3)	Pembrolizumab (anti-PD-1 mAb)	I	Relapsed or refractory diffuse large B cell lymphoma	NCT03340766	Recruiting
Blinatumomab (CD19 x CD3)	Pembrolizumab (anti-PD-1 mAb)	I/II	Recurrent of refractory acute lymphoblastic leukemia	NCT03512405	Not yet recruiting
Blinatumomab (CD19 x CD3)	Pembrolizumab (anti-PD-1 mAb)	I	Pediatric and young adult patients with relapsed or refractory acute leukemia or lymphoma	NCT03605589	Not yet recruiting
BTCT4465A (CD20 x CD3)	Atezolizumab (anti-PD-1 mAb)	I	Chronic lymphocytic leukemia, Non-Hodgkin lymphoma	NCT02500407	Recruiting
HER2Bi (HER2 x CD3)	Pembrolizumab (anti-PD-1 mAb)	I/II	Metastatic breast cancer	NCT03272334	Recruiting
HER2Bi (HER2 x CD3)	Pembrolizumab (anti-PD-1 mAb)	II	Prostate cancer	NCT03406858	Recruiting
IMCgp100 (gp100 x CD3)	Durvalumab (anti-PD-L1 mAb) tremelimumab (anti-CTLA4 mAb)	I	Malignant melanoma	NCT02535078	Recruiting
MGD007 (gpA33x CD3)	MGA012 (anti PD-1 mAb)	I/II	Relapsed or refractory metastatic colorectal cancer	NCT03531632	Recruiting
MGD009 (B7-H3 x CD3)	MGA012 (anti PD-1 mAb)	I	Relapsed or refractory cancer	NCT03406949	Recruiting
REGN1979 (CD20 x CD3)	REGN2810 (anti-PD-1 mAb)	I	Lymphoma	NCT02651662	Recruiting
Vanucizumab (Ang-2 x VEGF-A)	Selicrelumab (anti-CD40 mAb)	I	Advanced/metastatic solid tumors	NCT02665416	Recruiting
Vanucizumab (Ang-2 x VEGF-A)	Atezolizumab (anti-PD-L1 mAb)	I	Neoplasms	NCT01688206	Completed
RO6958688/RG7802 (CEA x CD3)	Atezolizumab (anti-PD-L1 mAb)	I	Advanced/metastatic solid tumors	NCT02650713	Recruiting
RO6958688/RG7802 (CEA x CD3)	Atezolizumab (anti-PD-L1 mAb)	I/II	Metastatic non-small-cell lung cancer	NCT03337698	Recruiting
RO7082859 (CD20 x CD3)	Atezolizumab (anti-PD-L1 mAb)	I	Relapsed refractory B non-Hodgkin's lymphoma	NCT03533283	Recruiting

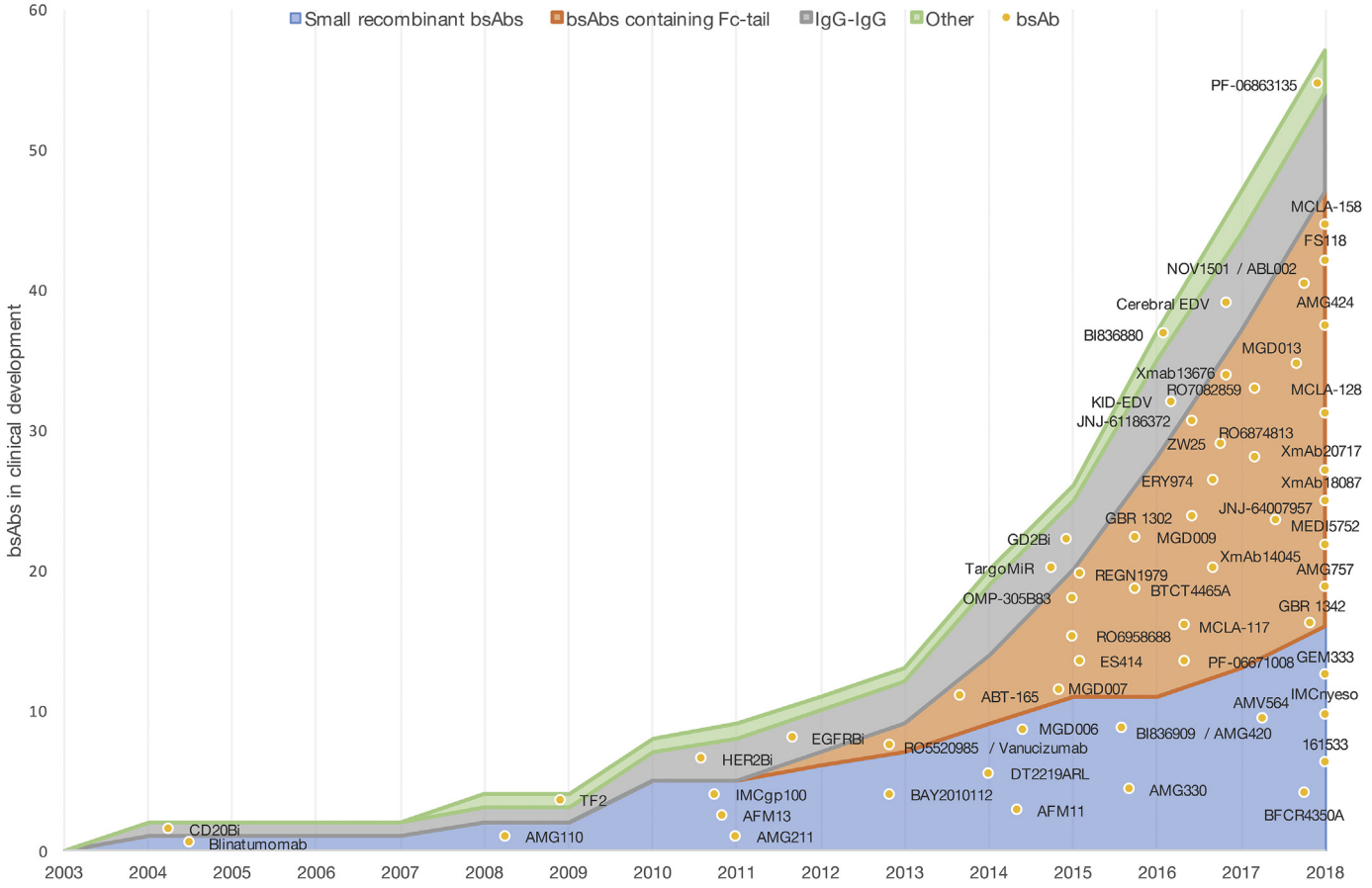

Fig. 4. BsAbs in development and registered in clinical trials at ClinicalTrials.gov in cancer patients. Lines display the number of constructs used per year and the bsAbs are displayed as dots. Their location in the chart indicates the construct used and the starting date of their first clinical trial.

also used to reduce cytokine release syndrome (Lee et al., 2014; Maude et al., 2014).

Recently, in humanized mice bearing a B cell lymphoma, pretreatment with an anti-CD20 antibody led to decreased toxicity after administration of a CD20- and CD3-targeting CrossMab bsAb, as measured by cytokine levels (Bacac et al., 2018). In that study design, the B cells in the peripheral blood and secondary lymphoid organs are depleted by the pretreatment, thus preventing their undesired activation and avoiding cytokine release by the immune-cell engaging bsAb (Bacac et al., 2018).

In addition, a recent study with a syngeneic mouse tumor model has shown a difference in distribution of HER2-targeting bsAbs with different affinity for CD3 (Mandikian et al., 2018). High affinity for CD3 reduced the systemic exposure and shifted uptake towards lymphoid tissues (Mandikian et al., 2018). Another study showed that the side effects of a bsAb engaging CD3 and C-type lectin-like molecule-1 are dependent on the CD3 affinity: the high-affinity variant induced high levels of cytokine release in cynomolgus monkeys (Leong et al., 2017).

These findings highlight the need for extensive pharmacokinetic studies of novel constructs like bsAbs, for example by means of molecular imaging. The design of bispecific antibody constructs is a challenge because the biodistribution of the drug is determined by both parts of the construct in combination with all other pharmacodynamics properties of the construct. Although there are many ways to measure pharmacokinetics of new drugs, molecular imaging the only non-invasive way.

Molecular imaging studies could be used to make predictive models for the pharmacokinetics of parts of bispecific constructs and develop optimal dosing strategies. This is especially relevant for all the differing constructs that have yet to be evaluated in clinical trials. An example of molecular imaging used for pharmacokinetics research is the development of a zirconium-89 labeled AMG211 tracer for positron emission tomography (Waaijer et al., 2018). AMG211 is a BiTE targeting CEA and CD3. In a phase I trial with patients with advanced gastrointestinal adenocarcinomas, metastases were imaged using this approach. There was heterogeneous tumor uptake within and between patients as well as CD3-specific uptake in lymphoid tissue (Moek et al., 2019).

8. Conclusion

As evidenced by the clinical trials evaluating these drugs, there is major interest in bsAbs as a treatment for cancer given. One bsAb is currently used in clinical practice, but none are undergoing phase 3 clinical trials for the treatment of cancer. Most of these bsAbs under evaluation have the same mechanism of action: the engagement of immune cells with tumor cells. For delivering payloads, the enthusiasm for using bsAbs seems to have been tempered due to the advent of facile conjugation methods such as click-chemistry. Preclinical studies suggest that antitumor efficacy of immune-cell engaging bsAbs will increase when combined with immune modulators such as anti-PD1 and anti-PD-L1 antibodies. The first clinical results confirm this, but more data is needed. The differing and novel constructs of bsAbs that will enter clinical trials also constitute a strong argument for the use of molecular imaging to reveal its in-vivo behavior. In recent history, the bsAb has been a versatile tool but besides blinatumomab it has not yet resulted in a clinical breakthrough. However, due to the increasing ease of production and their unique mechanisms of action, bsAbs can potentially be tailored to become a valuable addition to the oncology arsenal.

Conflict of interest statement

E.G.E.d.V. reports consulting/advisory fees from Sanofi, Pfizer, Daiichi Sankyo, NSABP and Merck, and research funding from Amgen, Genentech, Roche, Chugai Pharma, Synthon, CytomX Therapeutics, Nordic Nanovector, Regeneron, G1 Therapeutics, AstraZeneca, Radius Health, Servier and Bayer; all payments to the institution.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pharmthera.2019.04.006.

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