BISPECIFIC ANTIBODIES FOR T-CELL BASED IMMUNOTHERAPY

EDITED BY: Brian H. Santich, Nai-Kong Cheung and Christian Klein
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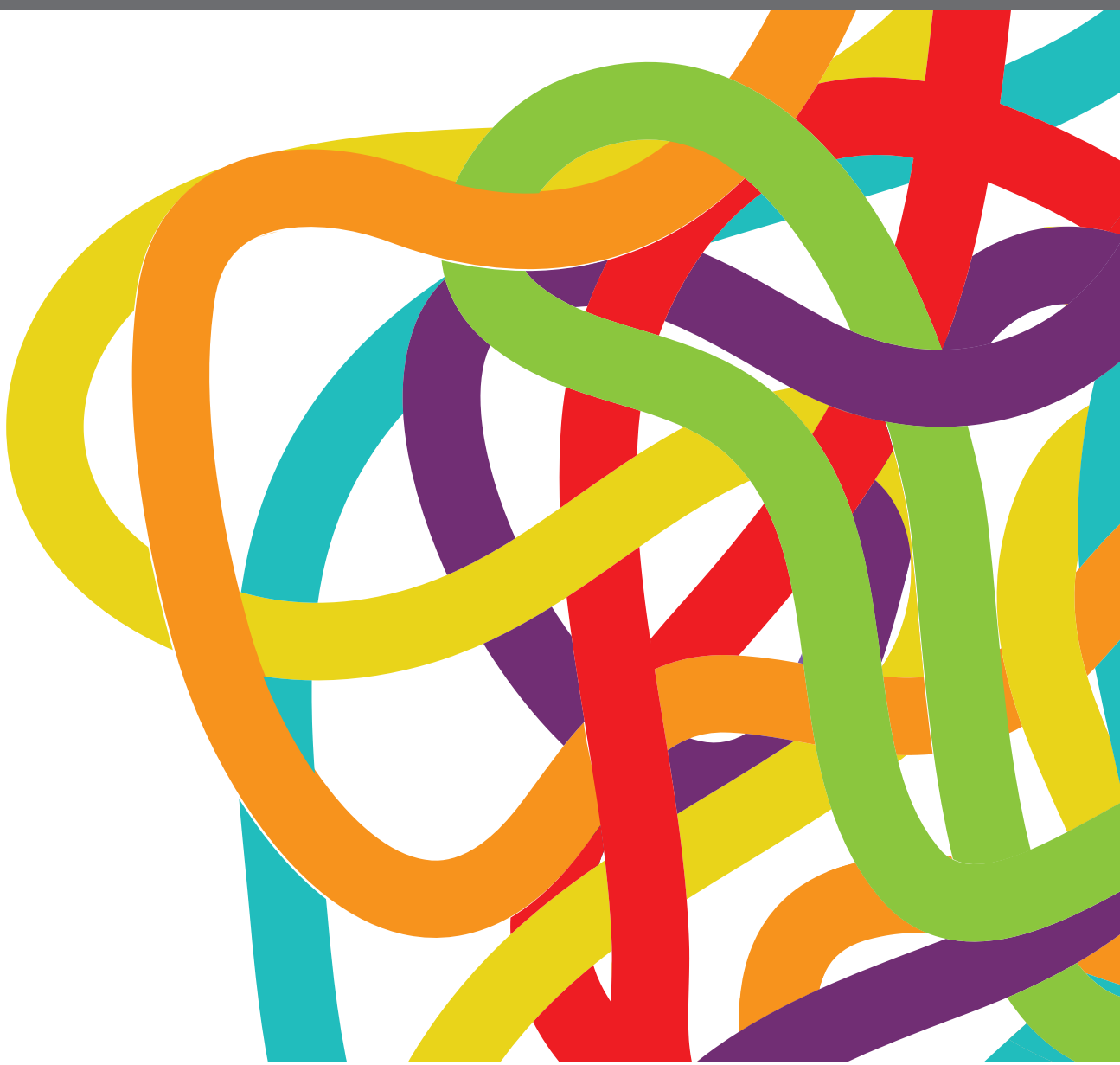

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BISPECIFIC ANTIBODIES FOR T-CELL BASED IMMUNOTHERAPY

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EDITORIAL
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Editorial: Bispecific Antibodies for T-Cell Based Immunotherapy

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Editorial on the Research Topic

Bispecific Antibodies for T-Cell Based Immunotherapy

BISPECIFIC ANTIBODY DESIGNS

To date, the FDA and EMA have approved bispecific antibodies (BsAb) using two different designs: the tandem-single-chain variable fragment (scFv) [blinatumomab (1)], and the heterodimeric IgG-molecule [emicizumab (2)]. However, more than 100 BsAb formats have been described in the literature, with varying molecular shapes, sizes, and valencies (3). While developmental considerations will always be an important decision, ultimately it is the functional properties of the design which dictate efficacy and safety. Vafa and Trinklein provide a valuable discussion of this subject, especially as it relates to epitopes for T-cell engagement. Of particular interest is the concept of decoupling cytokine release from anti-tumor cytotoxicity, thereby limiting the impact of cytokine release syndrome (CRS) and potentially permitting substantial increases in the maximum tolerated dose (MTD). Given the number of clinical trials which report CRS as the dose-limiting toxicity (4), such approaches, if confirmed in the clinic, could provide significant clinical benefit, and may also improve other CRS-inducing immunotherapies such as CAR-T cell therapy. Complementing this approach, Lum et al. have developed an alternative way to administer BsAbs by premixing or "arming" T-cells with BsAb ex-vivo prior to administration. This substantially reduces the total administrated BsAb dose, while still providing potent anti-tumor activity, as demonstrated in Dr. Lum's recent work targeting CS-1. Whether these approaches will succeed in a clinical setting remains to be seen.

Alternatively, work from De Luca et al. exemplifies how T-cell engaging BsAbs can be designed without CD3 targeting. Instead, De Luca and colleagues designed a trimeric format that localized IL-2 and TNF to CAIX-expressing tumors, with the TNF cytokine used both as an immune cell agonist and a multimerization tag for the protein itself. Doing so allowed them to take advantage of a greater avidity when binding to immune cells (trimeric vs monomeric) without increasing the protein complexity through additional multimerization domains or higher affinity interactions.

It is not currently clear how many more antibody designs will eventually receive clinical approval; however, as we learn more about protein design and engineering, newer and more advanced formats will become available, and hopefully improve the bispecific antibody landscape at large. However, as long as safety and potency remain the most important endpoints, future optimizations should remain focused on cytokine release, T-cell activation and cytotoxicity.

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TREATMENTS FOR B-CELL MALIGNANCIES: MYELOMA AND LYMPHOMAS

B-cell malignancies remain one of the model diseases for T-cell BsAb or adoptive T-cell immunotherapies, with the only FDA/EMA approvals so far occurring in leukemias and lymphomas. Due to the highly lineage restricted protein expression of many of B-cell tumor antigens (CD19, CD20, CD22, etc) as well as the treatable side effects of short and long-term B cell aplasia, therapeutics directed at B-cell malignancies have generally seen more clinical successes than those against solid tumors. However, as reviewed by both Lejeune et al. and Caraccio et al., the presence of safe and specific tumor antigens has not made the treatment of these diseases simple, particularly for multiple myelomas (MM) and indolent Non-Hodgkin lymphomas (NHL), which both remain largely incurable for most patients.

Multiple myelomas have offered several targets for T-cell BsAb treatment, such as CD38, CD138 or BCMA. Among these, BCMA is generally considered the most promising, due to its relative absence on non-lymphoid tissues, stem cells, or T-cells, and has recently seen the approval of an antibody-drug conjugate (belantamab mafodotin-blmf). Consequently, the majority of ongoing clinical trials for the treatment of MM have focused on BCMA, as reviewed by Dr. Caraccio. Non-Hodgkin lymphomas typically express common B-cell antigens, such as CD19 and CD20, two targets with approved antibody (CD19: tafasitamab; CD20: rituximab/obinutuzumab, ofatumumab) or BsAb therapeutics (CD19: blinatumomab). Like MM, ongoing clinical trials are exploring a multitude of BsAb formats, and some groups are even exploring combining therapies with other modalities, such as immune checkpoint inhibition (ICI), immunomodulatory imide drugs (ImIds) or antibody-drug conjugates (ADCs).

Given the difficulty in treating solid tumors, B-cell malignancies such as MM and NHL, may be the next indications where BsAb therapies provide significant clinical impact. With the enormous diversity of antibody formats being tested and CD3 epitopes being targeted, once phase I trials are completed it will be interesting to compare their safety profiles in addition to their relative anti-tumor effect.

COMBINATION TREATMENTS

Many groups have also begun studying combination therapies as an alternative method to overcome the limits of BsAb monotherapy, seeking to both improve the treatment efficacy and response duration. As demonstrated by Sam et al. combining T-cell engaging BsAbs with checkpoint blockade therapy, especially anti-PD-L1 immune checkpoint inhibitors, remains one of the most promising combinations to date. In addition to both PD-1 and PD-L1 being upregulated after treatment with BsAbs, both targets have multiple approved antibodies, greatly simplifying the clinical strategy for combination studies. Additionally, both T-cell BsAbs and ICI therapies have suffered from separate but potentially complementary limitations. T-cell BsAbs have struggled to treat solid tumors, while ICI therapies have had major successes treating lung cancer, colon cancer and melanoma. By contrast, ICI therapies are thought to require some kind of pre-existing immune infiltration, PD-L1 upregulation or T-cell immunity, with little efficacy in patients with so called "cold" or non-inflamed tumors (5), while T-cell BsAbs appear able to provide each of these functions quite effectively preclinically (6). It is therefore imaginable that T-cell BsAb therapy could be used to inflame an otherwise "cold" tumor and sensitize it to ICI therapy. It should be noted that with any synergy in efficacy comes the risk of synergy in toxicity, however this should be resolvable through appropriate dosing and treatment scheduling. Which targets and tumor types are most sensitive to such a combination therapy remains to be seen, however, judging by the study from Sam et al., MSI colon cancer appears very promising.

In summary, BsAb studies over the last several years have demonstrated compelling preclinical and early phase clinical data. This Research Topic explored many of these concepts and also proposed several new strategies for treating cancer with BsAbs. Going forward we hope to see each of these advance our understanding of T-cell immunotherapy and hope that some provide a much-needed improvement in clinical outcomes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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A Novel Fully-Human Potency-Matched Dual Cytokine-Antibody Fusion Protein Targets Carbonic Anhydrase IX in Renal Cell Carcinomas

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Certain cytokines synergize in activating anti-cancer immunity at the site of disease and it may be desirable to generate biopharmaceutical agents, capable of simultaneous delivery of cytokine pairs to the tumor. In this article, we have described the cloning, expression and characterization of IL2-XE114-TNF^{mut}, a dual-cytokine biopharmaceutical featuring the sequential fusion of interleukin-2 (IL2) with the XE114 antibody in scFv format and a tumor necrosis factor mutant (TNF^{mut}). The fusion protein recognized the cognate antigen (carbonic anhydrase IX, a marker of hypoxia and of renal cell carcinoma) with high affinity and specificity. IL2-XE114-TNF^{mut} formed a stable non-covalent homotrimeric structure, displayed cytokine activity in *in vitro* tests and preferentially localized to solid tumors *in vivo*. The product exhibited a partial growth inhibition of murine CT26 tumors transfected for carbonic anhydrase IX. When administered to *Cynomolgus* monkey as intravenous injection, IL2-XE114-TNF^{mut} showed the expected plasma concentration of ∼1,500 ng/ml at early time points, indicating the absence of any *in vivo* trapping events, and a half-life of ∼2 h. IL2-XE114-TNF^{mut} may thus be considered as a promising biopharmaceutical for the treatment of metastatic clear-cell renal cell carcinoma, since these tumors are known to be sensitive to IL2 and to TNF.

Keywords: immunotherapy, antibody-cytokine fusion proteins, IL2, TNF, EDA domain of fibronectin, CAIX

INTRODUCTION

Antibody-cytokine fusions (also called "immunocytokines") represent an emerging class of engineered cytokine products, that may display a superior anti-cancer activity as a consequence of a preferential accumulation at the tumor site, helping spare normal organs (1). An increased density of lymphocyte within the tumor mass typically correlates with a better prognosis, both in mice and in cancer patients (2–5). The targeted delivery of cytokines to the tumor environment may increase the intratumoral density of T-cells and NK cell (2, 4, 6). In this context, IL2 and interleukin-12 (IL12) have proven to be particularly attractive payloads for antibody-based delivery applications

(7–9), as these agents can potently activate T-cells and NK cells.

Antibody-cytokine fusions with tumor-homing properties are likely to display their therapeutic action and to increase the therapeutic index of the corresponding cytokine payload as a result of a specific activation and proliferation of tumor-resident CD8+ T cells and of NK cells, which recognize malignant structures (2, 3). For some pro-inflammatory cytokine payload (e.g., IL12), it has been shown that an antibody-based targeted delivery to the tumor may increase therapeutic activity by at least 20-fold, compared to the recombinant cytokine counterpart (10).

Antibody-cytokine fusion proteins in clinical trials for the treatment of cancer include various antibody-IL2 fusions [e.g., hu14.18-IL2 (11), huKS-IL2 (12), L19-IL2 (13), F16-IL2 (14), CEA-IL2v (15), NHS-IL2 (16), DI-Leu16-IL2 (17)], as well as fusions with TNF [e.g., L19-TNF (18)] and with IL12 [BC1-IL12 (19) and NHS-IL12 (20)]. More recently, scientists at Glycart-Roche have described a novel antibody fusion with human 4-1BBL, which has started clinical trials (21). The most advanced product may be represented by a combination of L19-IL2 with L19-TNF, which is currently being investigated in Phase III clinical trials (NCT02938299 and NCT03567889) for the treatment of fully-resectable Stage IIIB, C melanoma (22).

IL2 and TNF are representative examples of cytokines which work well when used in combination. Our group already described in 2010 that the simultaneous administration of L19-IL2 and L19-TNF in an immunocompetent mouse model of neuroblastoma was more active than the products given as single agents. Indeed, the combined use of the two products cured the majority of mice (23). A synergistic benefit for L19-IL2 and L19-TNF was also observed for intralesional administration procedures in mouse models of cancer (24). This work paved the way for the execution of a Phase II clinical trial in melanoma patients (22), which has then triggered Phase III study programs in Europe and in the United States. Tumor necrosis factor and IL2 are synergistic and complementary also from a pharmacodynamic viewpoint. TNF damages the tumor endothelium leading to a rapid hemorrhagic necrosis of the neoplastic mass (4, 6, 25), while IL2 mainly acts by activating NK cells and CD8+ lymphocytes (6, 24, 26).

The synergistic action of IL2 and TNF has stimulated research activities, aimed at incorporating both payloads into the same therapeutic product. We have previously described the production and anti-cancer activity of a dual-cytokine fusion protein (termed IL2-F8-TNF^{mut}), which exhibited a selective accumulation at the tumor site following intravenous administration and a potent anti-cancer activity, particularly against murine soft-tissue sarcomas (6). The therapeutic action of IL2-F8-TNF^{mut} could be potentiated by combination with immune checkpoint inhibitors (27).

Here, were report the cloning, production and characterization (*in vitro* and *in vivo*) of a novel fusion protein (termed IL2-XE114-TNF^{mut}), capable of recognizing carbonic anhydrase IX (CAIX) and of simultaneously displaying IL2 and a de-potentiated TNF mutant (TNF^{mut}). CAIX is a membrane protein, which is overexpressed in hypoxia conditions and in various cancer types, including renal cell carcinomas (RCC), urothelial, colorectal, stomach, pancreas, and other cancers (28, 29). This antigen is virtually undetectable in most normal adult tissues, exception made for certain gastrointestinal structures (29). CAIX has been targeted *in vivo* using both antibody- and small molecule-based products, showing interesting results in imaging studies (30–32).

The product was active *in vitro* and *in vivo* and may represent a candidate for the immunotherapy of renal cell carcinoma.

MATERIALS AND METHODS

Tumor Cell Lines

The human renal cell carcinoma cell line SKRC52 was kindly provided by Professor E. Oosterwijk (Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands). Transfected CT26-CAIX cells were prepared as previously reported (30). CHO cells, CTLL2 cells and L-M fibroblasts were obtained from the ATCC. Cell lines were received between 2017 and 2019, expanded, and stored as cryopreserved aliquots in liquid nitrogen. Cells were grown according the supplier's protocol and kept in culture for no longer than 14 passages. Authentication of the cell lines also including check of post-freeze viability, growth properties, and morphology, test for mycoplasma contamination, isoenzyme assay, and sterility test were performed by the cell bank before shipment.

Mice and Tumor Models

Six to eight-week-old female BALB/c nude mice were obtained from Janvier Labs. Tumor cells were implanted subcutaneously in the flank using 1×10^7 cells (SKRC52), 3×10^6 cells (CT26-CAIX).

Cloning, Expression, and Protein Purification

The fusion protein IL2-XE114-TNF^{mut} contains the antibody XE114 (31) fused to a mutated version of human TNFα (arginine to alanine mutation in the amino acid position 108 of the human *TNF* gene, corresponding to the position 32 in the soluble form) at the C-terminus by a 15-amino acid linker and to human IL2 at the N-terminus by a 12-amino acid linker (6). The gene encoding for the XE114 antibody and the gene encoding human TNF and human IL2 were PCR amplified, PCR assembled, and cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen) by a NheI/NotI restriction site as described previously (6).

The fusion proteins used in this study were expressed using transient gene expression in CHO cells as described previously (33, 34) and purified from the cell culture medium to homogeneity by Protein A (Sino Biological) chromatography.

In vitro Characterization

Purified proteins were analyzed by size-exclusion chromatography on a Superdex 200 increase 10/300 GL column on an ÄKTA FPLC (GE Healthcare, Amersham Biosciences). SDS-PAGE was performed with 10% gels (Invitrogen) under

reducing and non-reducing conditions. For ESI-MS analysis samples were diluted to about 0.1 mg/mL and LC-MS was performed on a Waters Xevo G2XS Qtof instrument (ESI-ToF-MS) coupled to a Waters Acquity UPLC H-Class System using a 2.1 × 50 mm Acquity BEH300 C4 1.7 μm column (Waters). Differential scanning fluorimetry was performed on an Applied Biosystems StepOnePlus RT-PCR instrument. Protein samples were diluted at 2 μM in PBS in 40 μL and placed in PCR tubes, assay was performed in triplicates. 5x SYPRO ORANGE (Invitrogen, stock 5000x) was added to samples prior to analysis. For thermal stability measurements, the temperature range spanned from 25 to 95°C with a scan rate of 1°C/min. Data analysis was performed in Protein Thermal Shift™ Software version 1.3. The temperature derivative of the melting curve was computed.

Affinity Measurements

Affinity measurements were performed by surface plasmon resonance using BIAcore X100 (BIAcore, GE Healthcare) instrument using a biotinylated CAIX coated streptavidin chip. Samples were injected as serial-dilutions, in a concentration range from 1 mM to 62.5 nM. Regeneration of the chip was performed by HCl 10 mM.

In vitro Biological Activities

The biological activity of TNF was determined by incubation with mouse LM fibroblasts, in the presence of 2 μg/mL actinomycin D (Sigma-Aldrich). In 96-well plates, cells (20,000 per well) were incubated in medium supplemented with actinomycin D and varying concentrations of recombinant human TNF or IL2-XE114-TNF^{mut}. After 24 h at 37°C, cell viability was determined with Cell Titer Aqueous One Solution (Promega). Results were expressed as the percentage of cell viability compared to cells treated with actinomycin D only.

The biological activity of IL2 was determined by its ability to stimulate the proliferation of CTLL2 cells. Cells (25,000 per well) were seeded in 96-well plates in the culture medium supplemented with varying concentrations of the fusion proteins. After incubation at 37°C for 48 h, cell proliferation was determined with Cell Titer Aqueous One Solution (Promega). Results were expressed as the percentage of cell viability compared to untreated cells.

Flow Cytometry

Antigen expression on SKRC52 cells was confirmed by flow cytometry. Cells were centrifuged and washed in cold FACS buffer (0.5% BSA, 2 mM EDTA in PBS) and stained with IL2-XE114-TNF^{mut} (final concentration 10 μg/mL) and detected with rat anti-IL2 (eBioscience 14-7029-85) followed by staining with anti-rat AlexaFluor488 (Invitrogen A21208). IL2-KSF-TNF^{mut} (specific for an irrelevant antigen) was used as negative control.

Immunofluorescence Studies

Antigen expression was confirmed on ice-cold acetone fixed 8 μm cryostat sections of SKRC52 and CT26-CAIX stained with IL2-XE114-TNF^{mut} and IL2-F8-TNF^{mut} (final concentration 5 μg/mL) and detected with rat anti-IL2 (eBioscience 14-7029-85) and anti-rat AlexaFluor488 (Invitrogen A21208). For vascular staining goat anti-CD31 (R&D AF3628) and anti-goat AlexaFluor594 (Invitrogen A11058) antibodies were used. IL2-KSF-TNF^{mut} (specific for an irrelevant antigen) was used as negative control. Slides were mounted with fluorescent mounting medium and analyzed with Axioskop2 mot plus microscope (Zeiss).

For *ex vivo* immunofluorescence analysis, mice were injected with 50–60 μg IL2-XE114-TNF^{mut}, IL2-F8-TNF^{mut}, or IL2-KSF-TNF^{mut} and sacrificed 24 h after injection. Organs were excised and embedded in cryo-embedding medium (Thermo Scientific) and cryostat section (10 μm) were stained using the following antibodies: rat anti-IL2 (eBioscience 14-7029-85) and anti-rat AlexaFluor488 (Invitrogen A21208). For vascular staining goat anti-CD31 (R&D AF3628) and anti-goat AlexaFluor594 (Invitrogen A11058) antibodies were used. Slides were mounted with fluorescent mounting medium and analyzed with Axioskop2 mot plus microscope (Zeiss).

Mice Therapy Studies

Mice were monitored daily and tumor volume was measured with a caliper (volume = length × width^2 × 0.5). When tumors reached a suitable volume (∼70–100 mm^3), mice were injected into the lateral tail vein with the pharmacological agents. Fusion proteins were dissolved in PBS, also used as negative control, and administered at 30 μg four times every 24 h. Results are expressed as tumor volume in mm^3 ± SEM and % mean body weight change ± SEM. For therapy experiments $n = 5$ mice/group.

Non-human Primate Study

The non-human primate study was performed in accordance with the Directive 2010/63/UE of the European parliament and of the council of 22 September 2010 for the protection of animals used for scientific purposes. Approval for the test site of experimentation: No. E 18-023-01. A total of 3 male naïve Macaca fascicularis (Cynomolgus monkey, Old Java monkey), ∼30 months at the time of allocation and estimated to weigh between 2.5 and 4.0 kg were used in this study. Test items were administered by bolus intravenous injection in the cephalic or saphenous vein, at a dose volume of 0.5 mL/kg body weight (corresponding to 0.1 mg/kg), over a period of ∼30 s. A flush with 1 mL of physiological saline was administered at the end of the bolus injection. The dose was administered to each animal on the basis of the body weight measured on the day of administration. Blood samples of ∼1 mL each were collected from the saphenous or cephalic vein (alternatively from other blood vessels) of all animals at approximately the following 7 time points: before dosing and at 1, 15, and 30 min and 1, 2, and 4 h after treatment. Samples were transferred into serum separator tubes, kept for 30 min in an upright position then centrifuged at room temperature (2,500 g for 10 min)

and the serum divided into two polypropylene tubes. Tubes were frozen within 90 min post blood sampling and stored at $-80 \pm 10°C$.

Pharmacokinetics Analysis

Fusion protein concentrations in serum were assessed by AlphaLISA. Briefly, Streptavidin Donor Beads were coated with biotinylated antigen (CAIX for IL2-XE114-TNF^{mut} or EDA for IL2-F8-TNF^{mut}). Acceptor Beads coated with an anti-TNF antibody were used for detection.

Statistical Analysis

Data were analyzed using Prism 7.0 (GraphPad Software, Inc.). Differences in tumor volume between therapeutic groups (until day 14, when $n = 5$) were evaluated with the two-way ANOVA followed by Bonferroni as post-test. $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

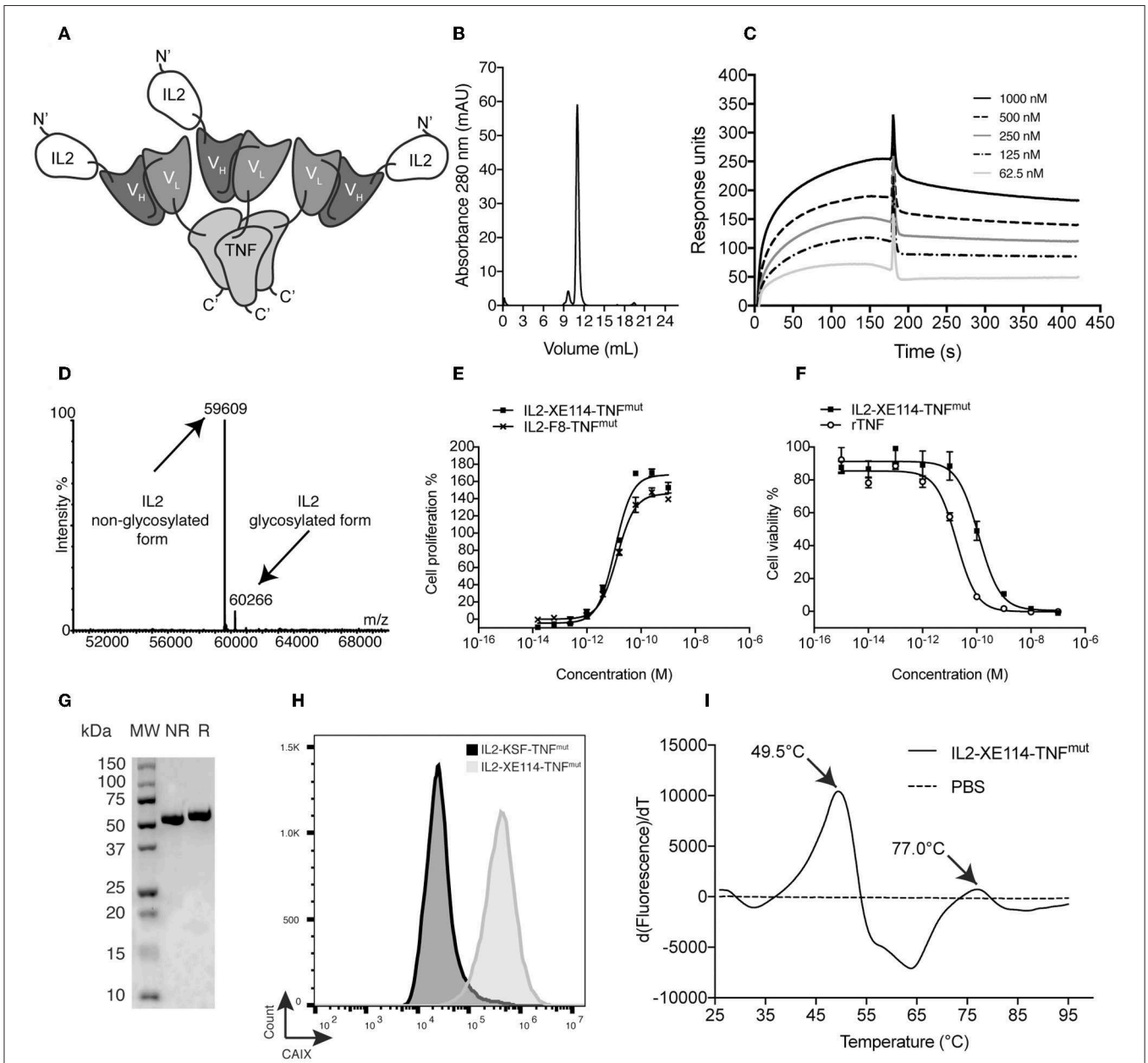

FIGURE 1 | Biochemical characterization of IL2-XE114-TNF^{mut}. **(A)** Schematic representation of the domain assembly of IL2-XE114-TNF^{mut} in non-covalent homotrimeric format. **(B)** Size exclusion chromatography profile. **(C)** BIAcore analysis on CAIX-coated sensor chip. **(D)** ESI-MS profile. **(E)** IL2 bioactivity assay on CTLL2 cells. **(F)** TNF bioactivity assay on L-M fibroblasts. **(G)** SDS-PAGE analysis; MW, molecular weight; NR, non-reducing conditions; R, reducing conditions. **(H)** Flow cytometric evaluation of CAIX expression by SKRC52 cells, detected with IL2-XE114-TNF^{mut}. **(I)** Differential Scanning Fluorimetry on IL2-XE114-TNF^{mut}.

RESULTS

Figure 1A depicts a schematic representation of a fully-human fusion protein (termed IL2-XE114-TNF^{mut}), featuring a sequential arrangement of IL2, a scFv fragment specific to CAIX [named XE114 (31)] and TNF (**Supplementary Figure 1**). The protein arrangement is reminiscent of the one previously described for the murine fusion protein IL2-F8-TNF^{mut} (6, 27) (**Supplementary Figure 2**), but here we used human payloads in order to facilitate clinical translational activities. The TNF moiety was de-potentiated by a single amino acid substitution (R431A), in order to achieve a similar cytokine activity for both IL2 and TNF moieties. ScFv(XE114) has previously been shown to recognize human CAIX with high affinity and kinetic stability (31, 35). IL2-XE114-TNF^{mut} was expressed in mammalian cells and could be purified on Protein A, since the scFv moiety featured a VH domain of the VH3 family (36, 37). The product formed stable non-covalent homotrimers in solution (**Figure 1B**), as TNF is a trimeric protein, and bound avidly to the cognate antigen in BIAcore assays (**Figure 1C**). The protein was mainly in a non-glycosylated form, but ∼10% of IL2-XE114-TNF^{mut} exhibited a molecular weight increase of 657 Dalton, as a result of O-linked glycosylation (**Figure 1D**). The product retained intact IL2 activity in an *in vitro* lymphocyte proliferation assay (**Figure 1E**), while TNF potency was reduced by ∼10-fold, as a result of a single amino acid substitution (6) (**Figure 1F**). A single band could be detected in SDS-PAGE analysis, both in reducing and in non-reducing conditions (**Figure 1G**). IL2-XE114-TNF^{mut} bound to SKRC52 renal cell carcinoma cell lines more intensely than IL2-KSF-TNF^{mut} (**Figure 1H**), which

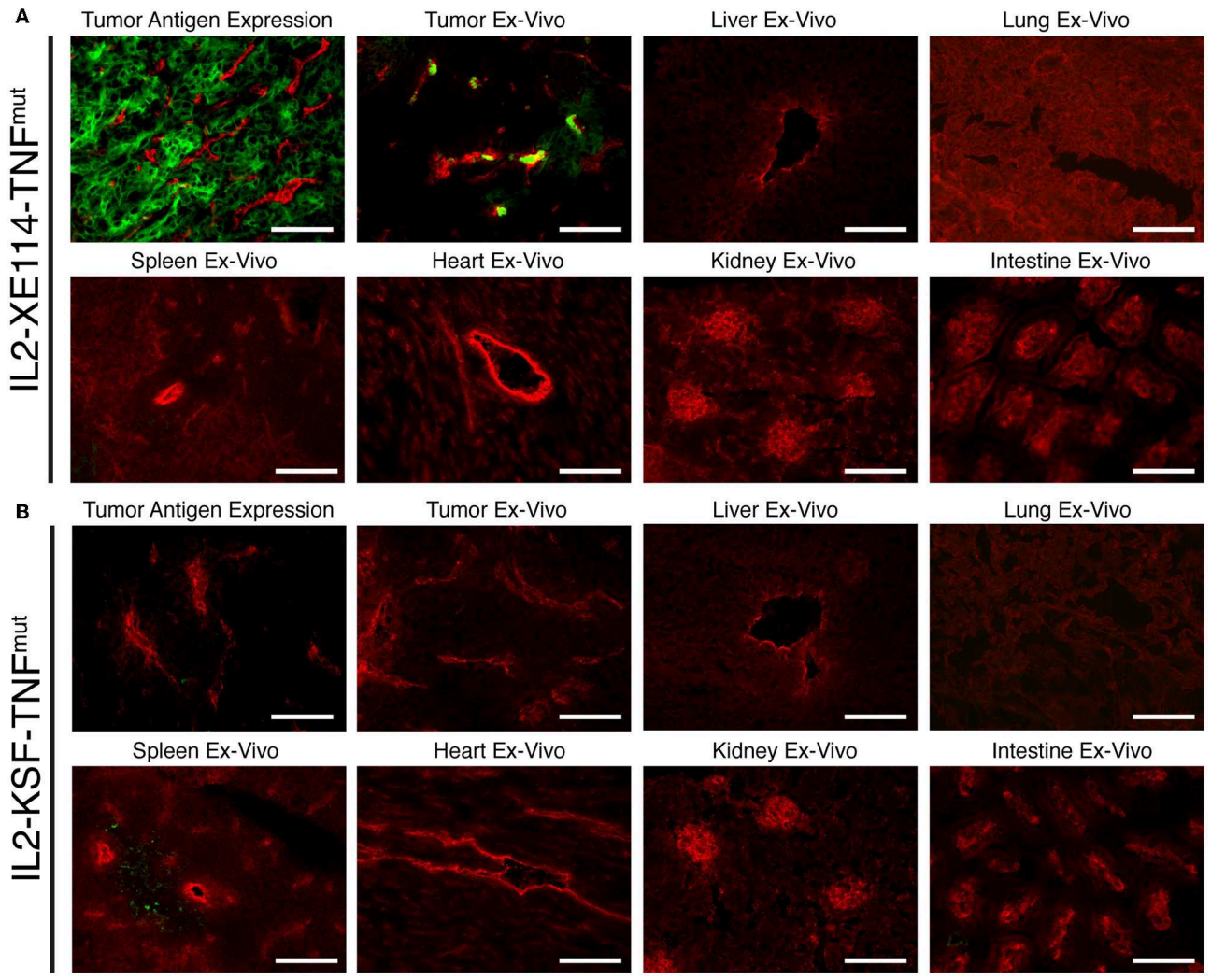

FIGURE 2 | Antigen expression and tumor targeting properties of IL2-XE114-TNF^{mut}. Microscopic fluorescence analysis of CAIX expression on SKRC52 tumor sections (**A,B** upper left) detected with IL2-XE114-TNF^{mut} or IL2-KSF-TNF^{mut} (negative control). Microscopic fluorescence analysis of organs from SKRC52 tumor bearing mice, 24 h after intravenous administration of IL2-XE114-TNF^{mut} **(A)** or IL2-KSF-TNF^{mut} **(B)**. Cryosections were stained with anti-IL2 (green, AlexaFluor 488) and anti-CD31 (red, AlexaFluor 594). 20x magnification, scale bars = 100 μm.

was specific to hen egg lysozyme and was chosen as a negative control of irrelevant specificity in the mouse (38) (**Supplementary Figure 3**). A multi-step denaturation profile was observed by differential scanning fluorimetry, with a first transition at 49.5°C (**Figure 1I**).

The tumor-homing properties of IL2-XE114-TNF^{mut} were first studied in nude mice, bearing subcutaneously-grafted human SKRC52 renal cell carcinomas (**Figure 2**). Organs were examined 24 h after intravenous administration of 60 μg of fusion protein, using an immunofluorescence procedure for the detection of the IL2 moiety. A homogenous antigen expression pattern was observed in an *in vitro* analysis of tumor sections. However, *ex vivo*, IL2-XE114-TNF^{mut} mainly localized to perivascular tumor cells and failed to homogenously stain the CAIX-positive tumor mass. Similar targeting behaviors have previously been reported for other antibody products, directed against cell surface tumor antigens (31, 39). No detectable antibody uptake could be seen in relevant normal organs (**Figure 2**). By contrast, no tumor staining and no tumor uptake could be observed for the IL2-KSF-TNF^{mut} negative control protein (**Figure 2B**).

In order to get a finer characterization of the tumor-targeting properties of our products, we compared IL2-XE114-TNF^{mut} and IL2-F8-TNF^{mut} (an analog specific to the alternatively-spliced EDA domain of fibronectin) (**Supplementary Figure 4**) in mice bearing murine CT26 tumors, that had been stably-transfected for CAIX expression on the cell surface (30). Both products exhibited a preferential accumulation at the tumor

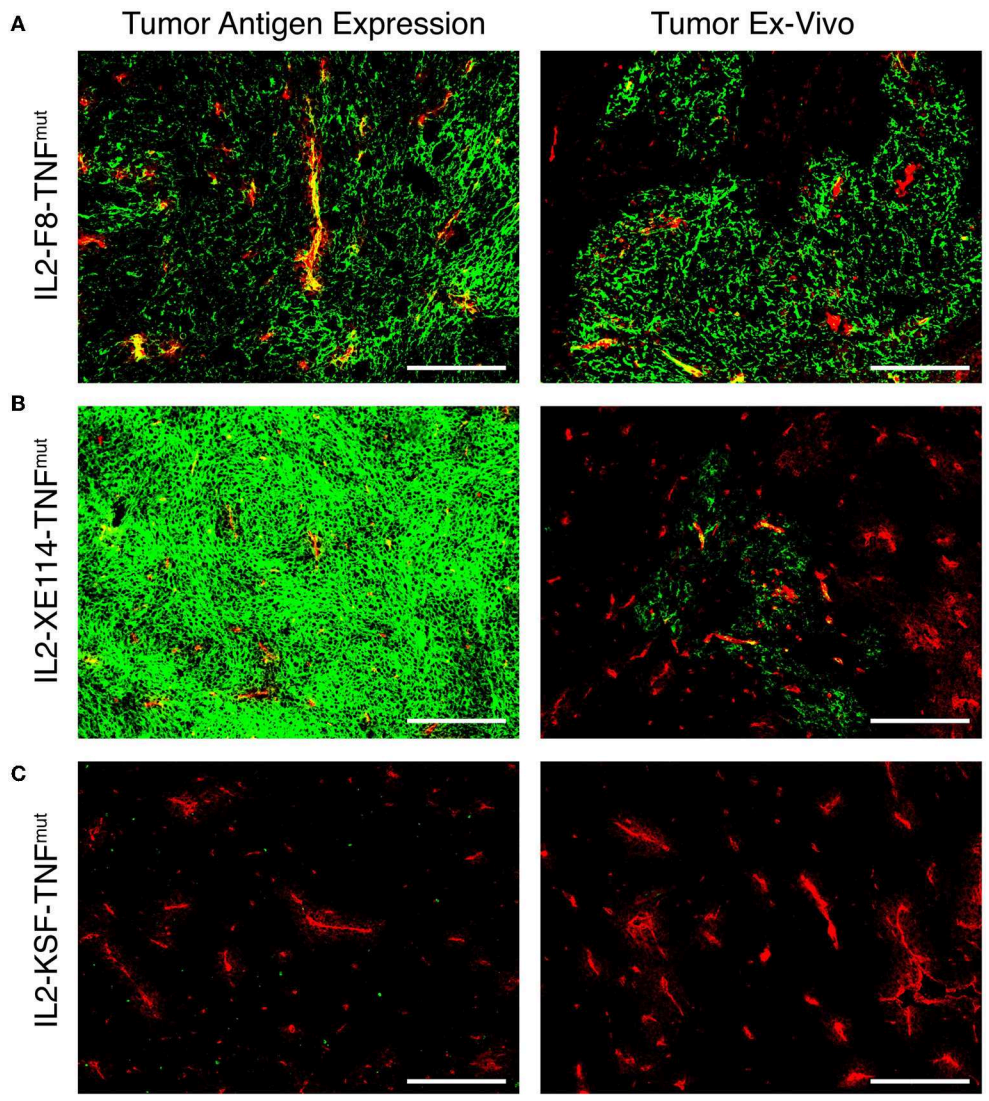

FIGURE 3 | Antigen expression and tumor targeting properties of IL2-XE114-TNF^{mut}. Microscopic fluorescence analysis of CAIX expression on CT26-CAIX tumor sections **(A–C)** detected with IL2-F8-TNF^{mut}, IL2-XE114-TNF^{mut}, or IL2-KSF-TNF^{mut} (negative control). Microscopic fluorescence analysis of tumors from CT26-CAIX tumor bearing mice, 24 h after intravenous administration of IL2-F8-TNF^{mut} **(A)**, IL2-XE114-TNF^{mut} **(B)**, or IL2-KSF-TNF^{mut} **(C)**. Cryosections were stained with anti-IL2 (green, AlexaFluor 488) and anti-CD31 (red, AlexaFluor 594). 10x magnification, scale bars = 200 μm.

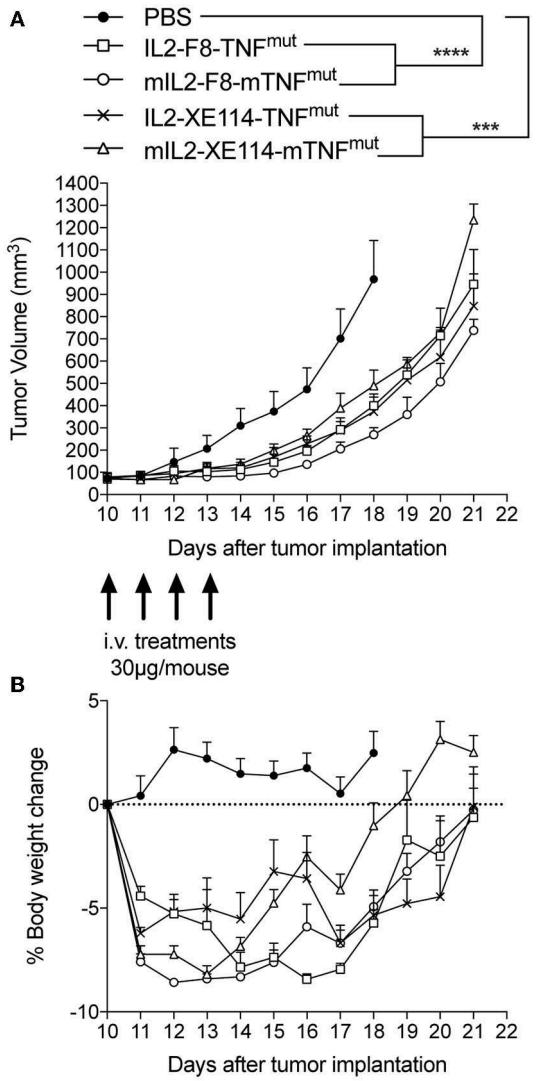

FIGURE 4 | Therapeutic performance of IL2-XE114-TNF^{mut} in BALB/c nude mice bearing CT26-CAIX tumors in comparison with IL2-F8-TNF^{mut} and their murine surrogates (mIL2-XE114-mTNF^{mut}, mIL2-F8-mTNF^{mut}). Treatment started when tumors reached a volume of 70–100 mm^3, mice were injected intravenously four times every 24 h with 30 μg of fusion proteins. Results are expressed as tumor volume in mm^3 ± SEM **(A)** and % mean body weight change ± SEM **(B)**. For therapy experiments $n = 5$ mice/group. $n = 4$ for group IL2-XE114-TNF^{mut} from day 15. $n = 4$ for groups mIL2-XE114-mTNF^{mut} and mIL2-F8-mTNF^{mut} from day 19. $n = 3$ for group mIL2-F8-mTNF^{mut} from day 20 (***$P < 0.001$, ****$P < 0.0001$).

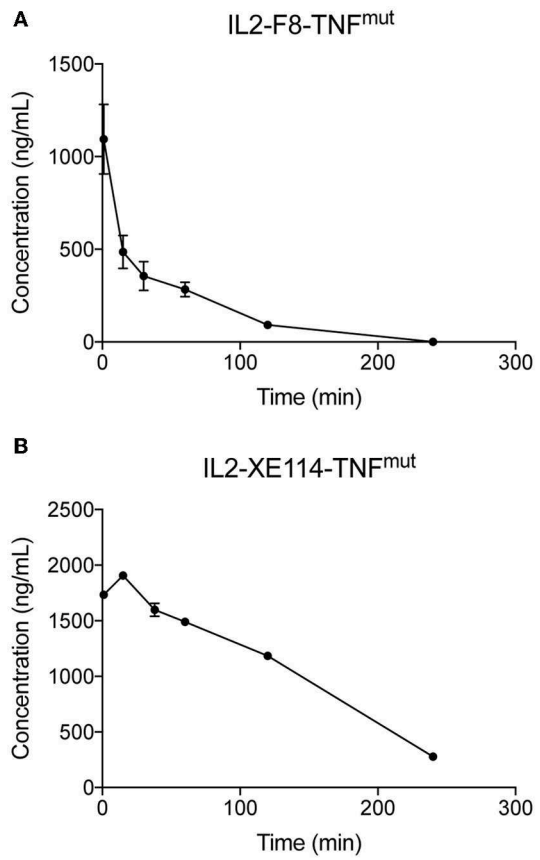

FIGURE 5 | Pharmacokinetics in cynomolgus monkeys. Pharmacokinetics were evaluated in one cynomolgus monkey per group injected once at the dose of 0.1 mg/kg of IL2-F8-TNF^{mut} **(A)** or IL2-XE114-TNF^{mut} **(B)**. Blood samples were collected before dosing and at 1, 15, and 30 min and 1, 2, and 4 h after treatment.

site, while IL2-KSF-TNF^{mut} failed to localize to the neoplastic mass. However, the IL2-F8-TNF^{mut} yielded a more homogenous pattern of tumor uptake compared to IL2-XE114-TNF^{mut}, in spite of the fact that CAIX was strongly expressed on all tumor cells (**Figure 3**).

We tested the therapeutic activity of IL2-XE114-TNF^{mut} and IL2-F8-TNF^{mut} in nude mice bearing CAIX-transfected CT26 tumors (**Figure 4**). Since the human TNF moiety is only partly active in mice, we also studied the therapeutic activity of the mIL2-XE114-mTNF^{mut} and mIL2-F8-mTNF^{mut} analogs, bearing an attenuated version of murine TNF (6, 27) (**Supplementary Figures 2**, **5**). A tumor-growth retardation was observed in this model, which lacked a functional set of T lymphocytes, but still retained natural killer (NK) cells (**Figure 4**). All products caused a transient reduction in body weight at the dose used (30 μg), which was below the 10% threshold.

Finally, we compared the pharmacokinetic profiles of IL2-XE114-TNF^{mut} and IL2-F8-TNF^{mut} in *Cynomolgus* monkey, after a single intravenous administration (0.1 mg/kg) (**Figure 5**). IL2-F8-TNF^{mut} showed a biphasic clearance profile, with a rapid loss of ∼2/3 of the protein from circulation, followed by a slower elimination phase. By contrast, IL2-XE114-TNF^{mut} exhibited a slower clearance profile, with a half-life of ∼2 h. This pharmacokinetic profile is similar to the one that we have previously observed for other antibody-cytokine fusion proteins, which have progressed to advanced clinical trials (13, 18, 40, 41).

DISCUSSION

In this work we have described the generation, the *in vitro* characterization and the *in vivo* validation of a novel

"potency-matched dual-cytokine antibody fusion protein" based on an anti-CAIX antibody fragment simultaneously fused to human IL2 and to a mutant version of human TNF. The product, named IL2-XE114-TNF^{mut}, could be expressed in mammalian cells and purified to homogeneity. The fusion protein was able to recognize CAIX both *in vitro* and *in vivo*, when tested on a human renal cell carcinoma cell line (SKRC52, which naturally express the antigen) and in a murine colon carcinoma cell line (CT26-CAIX, which had been transfected with the human antigen).

We have previously reported that the intralesional administration of two immunocytokine products (L19-IL2 and L19-TNF) was able to induce cancer remission both in mouse models of cancer (24) and in patients with stage IIIB/C melanoma (22). However, from an industrial prospective, the development of combination products may cause a duplication of activities and costs. By contrast, the opportunity of incorporate two cytokine payloads into the same antibody moiety may facilitate industrial development.

IL2- and TNF-based products have shown to be synergistically active against various type of malignancies (4, 6, 8, 22–25, 27, 42) by two distinct complementary mechanism of actions. On one hand, TNF is capable of inducing hemorrhagic necrosis and apoptosis of the tumor endothelial cells and also of cancer cells (4, 6, 25, 43, 44). On the other end, IL2 is able to promote a selective boosting of T cell and NK cell activity against cancer cells (6, 24, 26, 45).

The therapeutic activity of IL2-XE114-TNF^{mut} was evaluated in immunocompromised mice bearing human CAIX-transfected CT26 murine tumors. This mouse model, which lacked a functional set of T lymphocytes, but still retained NK cells, was chosen in order to avoid an immune response against the transfected human antigen. This may explain the reason why only an initial tumor growth inhibition was observed. We had previously reported that depletion studies in immunocompetent mice treated with IL2-F8-TNF^{mut} revealed a prominent role of CD4+ and CD8+ T cells in the cancer remission process (6).

Antibodies against cell surface antigens can recognize their cognate antigen with exquisite specificity, but often their penetration into solid malignancies can be suboptimal as a result of a slow extravasation rate due to their relatively large size (39, 46). Indeed, IL2-XE114-TNF^{mut} was found to preferentially localize to perivascular cells *in vivo*. Similar findings had been reported for antibodies specific to HER2 (39). Interestingly, it has been shown that a small molecule against CAIX could penetrate CAIX-positive tumors more efficiently compared to an antibody against the same target (31).

In spite of a suboptimal penetration in neoplastic lesions, IL2-XE114-TNF^{mut} revealed an acceptable clearance profile, with a half-life of ∼2 h in monkeys, which was more favorable compared to the one of IL2-F8-TNF^{mut}. This pharmacokinetic profile is comparable to the one previously observed for other cytokine-fusion proteins based on antibody-fragments (40, 47). By contrast, IL2-F8-TNF^{mut} exhibited a rapid clearance from circulation already at early time-points, possibly indicating a trapping of the F8 antibody in the liver, which was already observed for other F8-based immunocytokines (48).

Renal cell carcinoma (RCC) represents a rare condition that account for ∼2% of cancer deaths worldwide (49). The most prominent subtype of RCC (about 70%) is clear cell (ccRCC) (50). In most cases (70%) the tumor is confined to the kidney, but it may disseminate to regional lymph nodes and to visceral organs (50). Surgical resection is the primary treatment option for stage I-III RCC, but postsurgical recurrence is observed with a 5-year relapse rate of 30–40% in patients with stage II or III RCC (51). In the event of recurrence patients are typically treated with conventional chemotherapy (e.g., sunitinib), high dose IL2 or immune-checkpoint inhibitors (e.g., nivolumab and ipilimumab) (52, 53). A phase III clinical trials in patients with advanced renal-cell carcinoma showed that the combination of nivolumab and ipilimumab could increase the overall survival compared to sunitinib alone (53). However, this efficacy was observed only in a small portion of patients, for this reason novel therapeutics for the treatment of RCC may be required.

CAIX is strongly expressed in the majority of ccRCC. The antigen has been extensively validated as a target for ccRCC in preclinical studies and several antibodies against this antigen have been developed by our group and others (54, 55). An humanized monoclonal antibody, named G250 (56) has been used to validate CAIX as a cancer target by nuclear medicine in clinical studies (50, 57). The XE114 antibody fragment used in this study is a fully-human high-affinity antibody (31), which may be considered for clinical development of CAIX-targeted based therapeutics.

The product presented in this study (IL2-XE114-TNF^{mut}) was able to target CAIX in tumor bearing mice and showed a therapeutic effect in immunocompromised animals. Moreover, the favorable pharmacokinetic profile in monkey provide a rational for future clinical investigation. The targeted delivery of cytokine payloads to cell surface antigens may represent a valid alternative to the anchoring of pro-inflammatory payloads to the tumor extracellular matrix. To a certain extent, an immunocytokine able to selectively localize to tumor cell membrane may represent a functional equivalent to a "bispecific antibody" and may be capable of cross-link a tumor cell with a leukocyte (e.g., NK cell, T cell), which displays the cognate cytokine receptor on its surface.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Mice experiments were performed under a project license (license number 04/2018) granted by the Veterinäramt des Kantons Zürich, Switzerland, in compliance with the Swiss Animal Protection Act (TSchG) and the Swiss Animal Protection Ordinance (TSchV). The non-human primate study was performed in accordance with the Directive 2010/63/UE of the European parliament and of the council of 22 September 2010 for the protection of animals used for

scientific purposes. Approval for the test site of experimentation: No. E 18-023-01. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

RD and DN: conception and design, development of methodology, acquisition, analysis and interpretation of data, writing, review and revision of the manuscript, and study supervision. AV and SG: analysis and interpretation of data. BG, TO, BZ, AS, GB, and MM: technical support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01228/full#supplementary-material

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PERSPECTIVE
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Perspective: Designing T-Cell Engagers With Better Therapeutic Windows

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This perspective highlights the history and challenges of developing CD3-based bispecific T-cell engagers (TCEs) as cancer therapeutics as well as considerations and potential strategies for designing the next generation TCE molecules. The goal of this article is to raise awareness of natural T-cell biology and how to best harness the tumor cell killing capacity of cytotoxic T-cells with TCEs. In light of 30 years of concerted efforts to advance TCEs in early clinical development, many of the first-generation bispecific antibodies have exhibited lackluster safety, efficacy, and manufacturability profiles. As of January 2020, blinatumomab remains the only approved TCE. Many of the current set-backs in early clinical trials implicate the high-affinity CD3 binding domains employed and the respective bispecific platforms as potential culprits. The underlying conviction of the authors is that by taking corrective measures, TCEs can transform cancer therapy. Through openness, transparency, and much needed feedback from ongoing clinical studies, the field can continuously improve the design and effectiveness of next generation T-cell redirecting therapeutics.

Keywords: T-cell engager, bispecific, CD3 redirection, cancer, therapeutic window, cytokine release

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INTRODUCTION: HEEDING NATURE'S DESIGN

When considering the design of TCEs, it is important to appreciate the characteristics of immune-recognition and the biology of T-cells which we aim to redirect. Antibody-producing B-cells and T-cells are the effector cells that carry out the adaptive immune response and specifically recognize foreign proteins on infected or cancerous cells. T-cells recognize foreign peptides on infected or mutated cells through T-cell receptors (TCR) that bind foreign peptide-human leukocyte antigen complexes (pHLA) at low affinities ranging from 1 to 100 uM (1–3). Low affinity binding of the T-cell receptor to its cognate antigen is an important feature of the T-cell immune response. Consequently, the T-cell response is driven by avidity-based antigen recognition through multiple low-affinity TCRs (3–5). The TCR is a multi-protein complex that includes the CD3 subunits that translate cell surface antigen binding into an intracellular phosphorylation signaling cascade. These phosphorylation events culminate in the activation of transcription factors such as NFAT and NFkB that lead to increased expression of cytokines and effector proteins such as granzymes and perforin (5, 6). The intensity of signaling through TCR complexes ultimately determines T-cell fate, including cytolytic activity, proliferation, exhaustion, and apoptosis. Complementing pHLA:TCR complex signaling, both costimulatory and coinhibitory T-cell receptor pathways modulate the balance of controlled T-cell activation. It was through the understanding of these pathways that a

number of therapeutics (anti-CTLA4, anti-PD-1, and anti-PDL1) were developed to modulate T-cell activation against cancers expressing neoantigens and overcome the immune-suppressive microenvironment of tumors (7, 8).

A key observation relating to TCR signaling was highlighted by two different groups nearly two decades ago. These studies showed that induction of T-cell cytolytic activity does not require the formation of a stable and mature immunological synapse (9, 10). Importantly, Faroudi et al. noted that the activation threshold for target cell lysis was >1,000-fold more sensitive than the activation threshold for cytokine release, and that this difference was primarily due to differences in antigen concentration on the cell surface of target cells and the number of pHLA:TCR complexes formed. Together, these published studies established the dual threshold model of T-cell activation. The implications of this model along with the low affinity of natural TCR binding events are important considerations for determining the design parameters of T-cell engaging bispecific antibody therapeutics.

A BRIEF HISTORY OF CD3- BASED T-CELL ENGAGERS

A TCE is a protein that simultaneously binds through a target antigen on a tumor cell and CD3 on a T-cell to form a TCR-independent artificial immune synapse and circumvent HLA restriction. The earliest efforts using CD3 binding antibodies for T-cell activation date back the mid-1980's when studies of heteroaggregates of anti-CD3 (T3, from OKT3 hybridoma) showed anti-cancer cytotoxicity (11). The first published description of a bispecific TCE was of a rat isotype hybrid generated by Clark and Waldmann (12), who demonstrated targeted killing of TH-1 cells. Shortly after in 1990, a chemically conjugated TCE was created and used to demonstrate the first clinical proof-of-concept for treating malignant glioma in Japan (13). After a lull in clinical development of bispecifics due in large part to manufacturing complications, the field witnessed the clinical success of catamuxamab, an anti-EPCAMxCD3 mouse-rat hybrid bispecific administered intraperitoneally for malignant ascites (Fresenius Biotech, Germany, EMA approval in 2009, voluntarily withdrawn in 2017). Soon after, Micromet Inc. (Germany, USA) initiated trials for blinatumamab, a mouse anti-CD19xCD3 dual single chain variable fragment (scfv)-based bispecific, administered intravenously for acute lymphoblastic leukemia (ALL) (Amgen, CA, FDA approval in 2014).

While these early studies showed promising clinical efficacy, they were also hampered by severe dose-limiting toxicities primarily manifesting as cytokine release syndrome (CRS). This resulted in prohibitively narrow therapeutic windows and was due in large part to the anti-CD3 binding domains that were used. A comprehensive review of the literature shows that many early TCE drug developers relied on three primary mouse-derived anti-CD3 antibodies: OKT3, SP34, and UCHT1 (14–17). These original CD3 antibodies bind with a relatively high affinity in the single to low double-digit nM range. As described earlier, this is roughly 1,000-fold higher affinity than a natural pHLA:TCR interaction and likely has profoundly different effects on the activation of T-cells compared to natural signaling through the TCR.

After considering the limitations of first-generation TCEs in the context of the natural function of T-cells, we must re-think how we approach and engineer the next generation of bispecific T-cell engagers. Invoking the systems thinking motto of "optimizing subcomponents of a system does not necessarily optimize the overall system," it is worth reassessing our approach to multi-specific antibody development and the interdependencies of their structural and functional components. In a recent instructive review, Ellerman (18) provided a comprehensive perspective on the variables that can impact T-cell engagement. They include the antibody format, epitopes bound on CD3, membrane proximity of the epitope bound on the tumor antigen, target binding affinity, half-life, etc. (18). Mandikian (19) further highlighted importance of CD3 affinity of TCEs and their impact on tissue distribution. High affinity CD3-binders of HER- targeting TCEs were shown to distribute preferentially to secondary lymphatic tissues, reducing systemic exposure. In contrast, a high affinity tumor antigen binding domain was also suboptimal if rapidly internalized, with low residence time on the cell surface (19). In addition, when including an Fc to increase half-life of TCEs, a critical consideration is the elimination of Fc receptor interactions. Significant off-target toxicities (20, 21) and CRS that can arise from inadvertent cross-linking of standard Fc-containing bispecifics through adjacent Fc receptor-expressing cells (22), and active Fcs can potentially negatively impact *in vivo* efficacy (23). Arguably, when considering the aforementioned variables impacting TCE safety and efficacy, the failure of many early TCE therapeutic molecules may be a consequence of combining binding domains that were individually optimized but were not optimized to work together.

When considering the interdependencies of TCE structure and function, it is important to highlight the antibody format used and its impact on developability. A summary of commonly used formats for TCEs is shown in **Figure 1**. In addition to the biological complexities of initiating an artificial immune synapse, one of the key challenges with TCEs has been in the generation of fully human bispecific formats that are biophysically soluble, stable and manufacturable at large scale. Advances in antibody engineering since the 1990's have enabled an exponential increase in the number of formats and scaffolds that can be used in assembling bispecifics [**Figure 1** and reviewed in detail in (22, 24, 25)]. In these endeavors, the use of human sequences and the elimination of biophysical liabilities such as the amino acid residues that undergo post-translational modifications remain essential to producing therapeutic proteins. Specifically, TCE protein aggregates can have serious safety implications, given their potential to prematurely activate T-cells in the absence of target engagement. Enabling long-term stability of robust and non-immunogenic platforms will be key to the clinical advance of platforms to commercialization.

A challenge related to the biological mechanism of action of early TCEs derives from past patterns of thinking. Early TCE efforts were biased toward developing molecules with

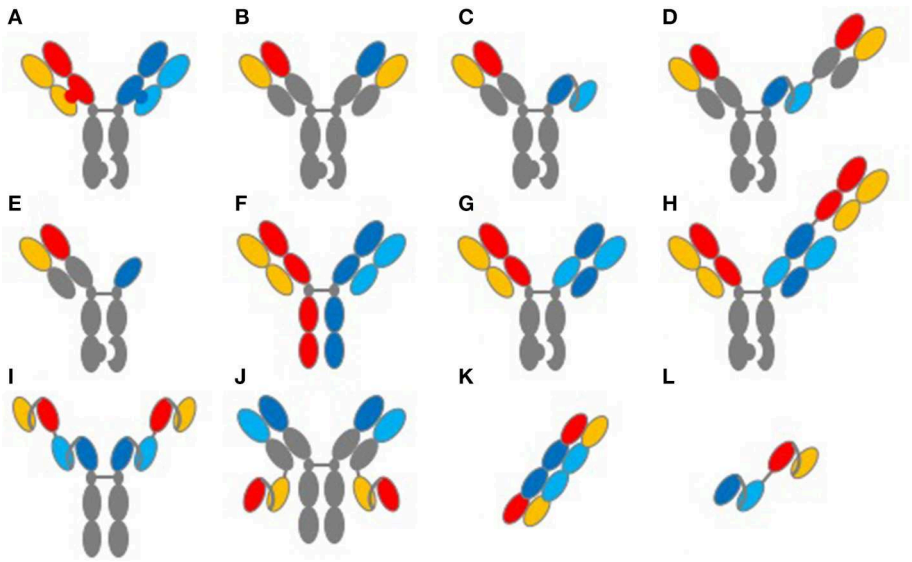

FIGURE 1 | Common structures of TCE proteins. This figure illustrates common molecular formats used to create TCE proteins. **(A)** knob-into-hole format for Fc and light-chain heterodimerization. **(B)** knob-into-hole format using a common light chain. **(C)** knob-into-hole triple-chain format, HC:LC Fab paired with scFv (Xencor) and **(D)** the 2+1 format including a second Fab (Xencor). **(E)** knob-into-hole triple-chain format, HC:LC Fab paired with heavy-chain only binding domain (Teneobio). **(F)** Fab arm exchange, DuoBody® (Genmab). **(G)** knob-into-hole Cross-MAb 1+1 format (Roche) and **(H)** knob into hole CrossMAb 2+1 format (Roche). **(I)** tetravalent scfv Fc fusion and **(J)** tetravalent HC:LC and scfv fusion (NV Cheung, MSKCC). **(K)** TandAb diabody (Affimed). **(L)** tandem scFv, first generation BiTE® format (Amgen).

the most potent cytotoxic activity based on *in vitro* cell-based assays without anticipating the biological consequences of high potency on cytokine release and T-cell exhaustion or depletion in the patient. These observations and safety concerns were summarized at a recent FDA-sponsored workshop focused on CD3 TCE safety assessment (26). Blinatumomab's small size and short half-life requires step-wise dosing (initial 9 μg/d followed by 28 μg/d by continuous infusion), which enables a steady Cmax to avoid neurotoxicity and CRS at higher concentrations (27). The second generation of TCEs include Fcs or other domains conferring half-life extension. Based on publicly reported adverse events and clinical holds in the last few years, the prospect of extending half-life with a high potency TCE could exacerbate serious adverse events associated with neurotoxicity and CRS. To address the complications associated with high potency anti-CD3 antibodies, companies like Xencor (Pasadena, CA) and Macrogenics (Gaithersburg, MD) mutated the SP34 anti-CD3 antibody to humanize and reduce binding affinity in efforts that demonstrated reduced cytokine release *in vitro* and *in vivo* (28, 29). Nevertheless, it remains to be determined whether reduced-affinity anti-CD3 TCEs will improve therapeutic window since the original SP34 anti-CD3 binding domain remains suboptimal in the clinic. Preventative measures for CRS have relied on pre- or co-medication with corticosteroids as well as anti-IL6R (tociluzimab) to ameliorate grade 3 and 4 adverse events. Whether such treatments also compromise the efficacy of TCEs is a matter of current debate.

THE NEXT GENERATION OF T-CELL ENGAGERS

Due to the limitations of the first and second generation TCEs that relied on re-purposing mouse-derived CD3 antibodies such as OKT3, SP34, and UCHT1, more recent efforts have focused on discovering new CD3 binders and adopting the principles of holistic design. **Figure 2** summarizes the design considerations for the CD3 binding domain in the context of the other binding domains of a TCE molecule. With these considerations in mind, the goal of new discovery efforts is to identify CD3 binding antibodies that are fully human and bind new epitopes on the CD3 complex with a range of affinities. Most importantly, these new CD3 antibodies are meant to be "fit-for-purpose," designed and functionally screened specifically for optimal behavior in TCE bispecific antibodies. Toward this goal, we at Teneobio (Newark, CA) have discovered numerous novel human anti-CD3 binding domains through sequence-based discovery of fixed light chain transgenic rats (30).

Based on the previous work of Faroudi et al. (9), our goal was to identify leads which preferentially trigger the cytolytic activity of T-cells and avoid the production and secretion of large quantities of pro-inflammatory cytokines. Characteristic of one of the CDR families (F2) was that its members uniquely bound a conformational epitope that recognizes the CD3δε heterodimer preferentially over CD3γε and over a wide range of affinities from low to high nanomolar (30). Importantly, in the context of human IgG heterodimeric bispecific antibodies, F2 family

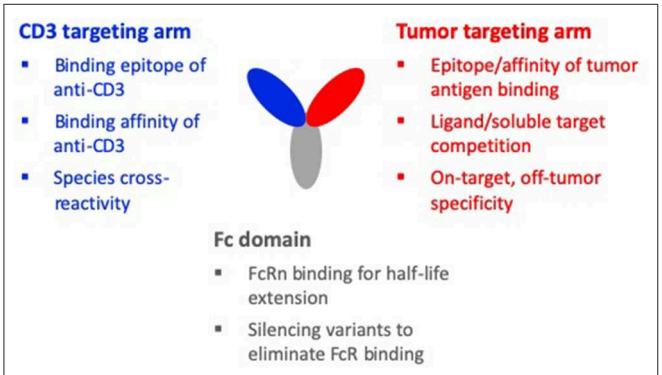

FIGURE 2 | Design considerations of a TCE molecule. The three binding domains of a typical TCE molecule are the CD3 targeting arm, the tumor targeting arm, and the Fc domain. When designing the CD3 targeting arm, important considerations are: the binding epitope, the binding affinity, and species cross-reactivity. For the tumor targeting arm, important considerations are: the epitope and affinity of tumor antigen binding, competition with soluble target or a ligand of the target, and on-target, off-tumor specificity. Considerations for the Fc domain are: maintaining FcRn binding for half-life and silencing variants to eliminate FcR binding, complement activity, and non-specific CD3 clustering and T-cell activation. For the holistic design of a TCE molecule, these design considerations must be made in the context of the interdependencies of the different domains.

members retained full efficacy against cancer target cells while demonstrating low levels of cytokine release (30). Consistent with our results, recent studies by Zuch de Zafra et al. (31) and Li et al. (32) also demonstrated that T-cell mediated cytotoxicity can be decoupled from cytokine release when using TCEs. Li et al. further showed that initial release of TNF from T-cells was the primary culprit driving CRS by triggering downstream proinflammatory cytokine release from monocytes. Moreover, Teneobio's F2 family members can preferentially activate CD8+ cells over regulatory T-cells (unpublished data). This differential activation is noteworthy and therapeutically relevant, given that Duell et al. (33) showed that blinatumomab (based on the anti-OKT3 scaffold) can activate Tregs and thereby inhibit T-cell proliferation and killing. Finally, unlike the first generation anti-CD3 TCEs, F2 family-based TCEs do not upregulate T-cell inhibitory receptors such as PD1 and CTLA4, which are hallmarks of T-cell exhaustion and/or anergy (unpublished data). This unique attribute of the F2 family binders is likely due to signaling intensity driven by CD3 affinity and the distinct binding epitope on CD3δε. Importantly, TCE developers using platforms based on OKT3 should take heed of the fact the OKT3 is apoptotic in the presence of IL-2 (34) and that clinical studies involving humanized OKT3 (teplizumab, hOKT3g1) to treat type I diabetes demonstrate that teplizumab induces T-cell exhaustion as well as apoptosis of CD8+ T-cells (35). These observations have obvious clinical relevance and pose potential liabilities when selecting OKT3-based binders for TCE platforms.

An additional consideration when designing a TCE with a better therapeutic window is whether decoupling cytotoxicity from cytokine release can impact maximal efficacy, especially for solid tumors. In theory, completely eliminating IFNγ production could minimize its anti-tumoral effects and dampen downstream immune stimulation from HLA class I upregulation (36). On the other hand, IFNγ can also upregulate PD-L1, posing unwanted tumoricidal resistance, necessitating PD-L1 blockade (37). The ideal level of cytokine production and how the pleiotropic effects of cytokines impact the efficacy of next generation of TCEs is the subject of current debate and will require further investigation in preclinical models and human patients.

Beyond identifying TCE-optimized CD3 binding domains, a number of companies are exploring alternative approaches to designing therapeutics which can reduce cytokine release and improve safety. Biotech companies like CytomX (South San Francisco, CA), Maverick Therapeutics (Brisbane, CA), and Amunix (South San Francisco, CA) have introduced proteolytic sites in their therapeutic molecules whereby local tumor cell proteases can cleave and conditionally activate the respective highly potent TCEs at the site of the tumor, potentially minimizing systemic toxicities. These various formats are currently in preclinical stages of development and undergoing IND-enabling studies. The success of these platforms will undoubtedly rely on their stability post-manufacturing and the retention of the conditionally activated bispecific at the tumor site with minimal diffusion that may impact on-target off-tumor cytotoxicity.

FUTURE OPPORTUNITIES AND CHALLENGES

Early clinical results and the new improvements to TCE design has spurred the discovery and clinical advance of 66 bispecific TCEs that are now in Phase I and Phase II studies as of January 7, 2020. Current clinical studies of TCEs span both hematological (39 trials) and solid tumors (34 trials), with over a hundred additional programs in preclinical development (personal communication with Paulina Szymanska, Beacon Target Therapies). Not surprisingly, most pharma and biotech companies are pursuing hematological cancers by targeting lymphocyte restricted tumor-associated antigens such as CD19, CD20, BCMA, CD33, and CD123. Importantly, as disclosed in the most recent American Society of Hematology (ASH) abstracts in December of 2019, a number of novel TCEs targeting BCMA and CD20 are showing favorable and complete responses in myeloma and lymphoma patients, respectively (38–42).

While the early clinical results with TCEs in hematological cancers are showing impressive efficacy, solid tumors represent a patient population that is 10-times larger with an even greater unmet medical need. One of the major goals in the field of TCE is effectively addressing solid tumors. To this end, multiple companies in pharma and biotech are pursuing TCEs targeting common, over-expressed solid tumor antigens such as HER2, PSMA, EPCAM, and CEA. Others are pursuing pHLA neoantigens as targets using T-cell receptor (TCR) protein scaffolds (e.g., Immunocore, UK) and TCR mimics comprising antibody scaffolds that recognize HLA-peptide complexes (Eureka Therapeutics, CA, Gritstone Oncology, CA). However, it is unlikely that TCEs can simply be applied to

solid tumors in the same way they are used in hematological cancers. Solid tumor cancers are fundamentally different diseases compared to hematological cancers (43). Unlike many of the B-cell targets whose expression is limited to the B cell lineage, the aforementioned solid tumor antigen targets are not exclusively restricted to tissues of origin associated with specific cancers. Therefore, TCEs targeting solid tumor-associated antigens must address safety concerns related to "on-target, off-tumor" activity in healthy tissues (26, 44). One way this is being addressed is with a bivalent CEA-targeting TCE (2+1 format) that preferentially targets high expressing CEA on solid tumors while avoiding low expressing primary cells (45). Another example is a HER2-targeting TCE that uses multi-valent avidity-based HER2 binding that biases activity to tumor cells with the highest antigen density (46). With this multi-valent antigen binding design, the low level of HER2 expression on cardiac cells and other healthy tissue is insufficient to induce T-cell engagement and activation in mouse models of HER2-positive breast cancer.

In addition to tumor specificity, other significant challenges in treating solid tumors with TCEs are overcoming the immunosuppressive tumor microenvironment (TME) and the physical barriers to cytotoxic T-cell trafficking and tumor penetration defined as the stroma (47). Solid tumors recruit immunosuppressive cells such as myeloid derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T-cells (Tregs), all of which inhibit the activity of cytotoxic T-cells. Therefore, the most effective use of TCEs in solid tumors will likely require using TCEs combined with agents such as checkpoint inhibitors and stroma disrupters that help to overcome the immunosuppressive TME and render an immune excluded or immune desert "cold" tumor into an inflamed "hot" one (48). In addition to checkpoint blockade, antibody agonists to co-stimulatory targets such as CD28 and CD137 as well as immune-activating cytokines such as IL-2 and IL-15 can promote the expansion of peripheral T-cells and lower the threshold for T-cell activation and are being investigated as ways to overcome immunosuppression in solid tumors. In this context, it is essential that TCEs have a favorable safety profile and broad therapeutic window when used in combination to address solid tumors.

Combination treatments that break the stroma barrier, comprising basement membrane, fibroblasts and the extracellular matrix, could enable T-cell penetration. These may involve the use of antibody drug conjugates or alpha-emitters to stroma cells, targeting fibroblast activation protein alpha (FAP-alpha), or the FGF and TGF-ß pathways (47, 49). Other approaches involve targeting stellate cells, hyaluronan, and secreted extracellular matrix (ECM) associated proteins (50, 51). A number of preclinical proof of concept studies show the feasibility of some of these aforementioned approaches [reviewed in (47)], which will be ripe for early clinical experimentation in combination with TCEs, pending favorable outcomes of current ongoing clinical trials (e.g., see clinical trials.gov for Phase I and II studies of sibrotuzumab (NCT02198274), Fresolimumab (NCT02581787), defactinib (NCT03287271), and AZD4547 (NCT01791985). Ultimately, we anticipate that TCEs with improved therapeutic windows may afford favorable synergies in solid tumor treatment with checkpoint inhibitors, stroma disrupters, targeted co-stimulatory agents or cytokines, and other modulators of the solid tumor microenvironment.

CONCLUDING REMARKS

The old alchemical phrase, "*In sterquiliniis invenitur*" translates to "in filth it will be found." Implicit in this message is that what you need most can be found in the mess where you least wish to look. This phrase can be applied to the clinical development of TCE therapeutics where biological complexity and clinical failures are ever-present challenges. Our industry could improve the transparency with which we share the details of failures in both TCE preclinical and clinical development. Not knowing the basis for such failures can delay faster and informed development of better TCEs. Openness and learning from both preclinical and clinical outcomes will enable continuous improvement in building better molecules for meaningful therapeutic benefits to patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Bispecific Antibodies for Multiple Myeloma: A Review of Targets, Drugs, Clinical Trials, and Future Directions

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Multiple myeloma (MM) is a plasma cell malignancy and the second most common hematological neoplasm in adults, comprising 1.8% of all cancers. With an annual incidence of ∼30,770 cases in the United States, MM has a high mortality rate, leading to 12,770 deaths per year. MM is a genetically complex, highly heterogeneous malignancy, with significant inter- and intra-patient clonal variability. Recent years have witnessed dramatic improvements in the diagnostics, classification, and treatment of MM. However, patients with high-risk disease have not yet benefited from therapeutic advances. High-risk patients are often primary refractory to treatment or relapse early, ultimately resulting in progression toward aggressive end-stage MM, with associated extramedullary disease or plasma cell leukemia. Therefore, novel treatment modalities are needed to improve the outcomes of these patients. Bispecific antibodies (BsAbs) are immunotherapeutics that simultaneously target and thereby redirect effector immune cells to tumor cells. BsAbs have shown high efficacy in B cell malignancies, including refractory/relapsed acute lymphoblastic leukemia. Various BsAbs targeting MM-specific antigens such as B cell maturation antigen (BCMA), CD38, and CD138 are currently in pre-clinical and clinical development, with promising results. In this review, we outline these advances, focusing on BsAb drugs, their targets, and their potential to improve survival, especially for high-risk MM patients. In combination with current treatment strategies, BsAbs may pave the way toward a cure for MM.

Keywords: BCMA, bispecific antibodies, CD38, clinical trials, high-risk disease, multiple myeloma, review

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INTRODUCTION

Multiple myeloma (MM) is the second most common hematologic malignancy in adults (1). In the United States in 2018, ∼30,770 patients were diagnosed with MM and 12,770 died from their disease, representing 2.9% of all cancer deaths (2). MM is characterized by a clonal expansion of malignantly transformed plasma cells in the bone marrow (BM). These cells produce an excess of monoclonal immunoglobulins, which are secreted into the blood and urine. Major complications in MM patients include tumor-induced bone lesions and associated pathological fractures, anemia, renal failure, and immunodeficiency, leading to impaired quality of life and decreased overall survival (3, 4).

Over the last few decades, novel drug classes such as immunomodulators (e.g., lenalidomide), proteasome inhibitors (e.g., bortezomib), histone deacetylase inhibitors (e.g., panobinostat), and monoclonal antibodies (mAbs) (e.g., daratumumab [anti-CD38]) have significantly improved the response rates and overall survival for MM patients (5, 6). Currently, the median overall survival for MM patients is 5 years (7). However, stratification by disease risk, according to the Revised International Staging System (R-ISS), reveals significant variability: 82% of low-risk, stage I patients survive 5 years, compared to only 40% of high-risk, stage III patients (7). While high-risk MM patients only account for 15 to 20% of newly diagnosed cases, these patients are often primary refractory to treatment or relapse early (8). Additionally, the majority of low-risk MM patients ultimately develop drug-resistant clones, become refractory to treatment and transition to high-risk disease (8–10). These findings underscore the need to identify MM patients who have active high-risk disease, as well as those who are likely to progress, and develop novel treatment strategies targeted at this population.

Bispecific antibodies (BsAbs) offer a promising immunotherapeutic approach for numerous malignancies including MM. Immune effector cell redirecting BsAbs commonly bind to a tumor cell antigen and CD3 on a T cell, resulting in T cell binding to the tumor cell, activation, and tumor cell lysis (11, 12). Since BsAbs directly stimulate CD3 and thus bypass the T cell receptor, they activate T cells independently from antigen presentation on major histocompatibility complex (MHC) class I. In addition, they have the ability to activate T cells in the absence of co-stimulation, bypassing the normal dependence on antigen presenting cells or cytokines and reducing the risk of anergy that accompanies TCR stimulation in the absence of a costimulatory signal (12–20).

Here, we review the potential of BsAbs in MM, with an emphasis on high-risk patients, although the benefits of BsAbs can extend to all MM patients. A brief introduction into MM is followed by an overview of current BsAb strategies. Next, novel BsAb developments and clinical trials for different MM targets are discussed. Finally, the future direction of BsAbs as a MM treatment modality is addressed, along with obstacles that need to be overcome.

ORIGINS OF MM AND FEATURES OF HIGH-RISK DISEASE

MM is a cancer of plasma cells, which are terminally differentiated B cells. Numerous hematologic malignancies result from the malignant transformation of B cells at different stages in their lifecycle. For instance, B cell leukemias usually arise from BM-residing pre-B cells; B cell lymphomas, from mature B cells that have migrated to lymph nodes; and MM, from long-lived, BM-residing plasma cells (**Figure 1A**). Malignant B cells and malignant plasma cells have key differences in their molecular architecture. Unlike malignant B cells, malignant plasma cells generally do not express the widely targeted cell-surface proteins CD19 and CD20, although up to 20% of patients have $CD20^{+}$ MM clones (23). As such, most MM patients cannot be treated with many of the newly-approved targeted therapies for B cell leukemias and lymphomas. This is in part reflected by the number of new drug approvals/indications, which was 56 for B cell leukemias/lymphomas and only 10 for MM in the past 4 years [(21, 22); **Figure 1B**].

MM develops from a pre-malignant condition known as monoclonal gammopathy of undetermined significance (MGUS), which progresses to smoldering MM (SMM) and MM at a rate of 1% per year (24). Virtually all known MM cases are preceded by MGUS, but the vast majority of MGUS cases never develop into MM. MGUS is characterized by low levels of serum M-protein (<3 g/dL) and <10% clonal BM plasma cells, whereas these levels are >3 g/dL and >10% for SMM, respectively (24). Importantly, the proliferation of malignant plasma cells in patients with MGUS and SMM is asymptomatic and these patients do not exhibit end-organ damage. MM patients are further staged into risk categories, which predict prognosis and treatment response. The most commonly used risk stratification model is the R-ISS, but several models exist (**Table 1**). With ongoing disease progression, MM patients ultimately develop aggressive, end-stage MM in the form of extramedullary disease or plasma cell leukemia. **Table 2** details emerging factors associated with high-risk MM.

Genetic and epigenetic events that initiate MM include chromosomal hyperdiploidy, translocations of chromosome 14 (bringing the strong immunoglobulin heavy-chain enhancer into the proximity of oncogenes), the dysregulation of cell cycle genes, abnormalities in signaling pathways, and alteration of DNA methylation (36–38). Further aberrations, including MYC overexpression and mutations in RAS oncogenes, amongst others, are associated with disease progression (34, 36). In contrast to the evolving genetic heterogeneity associated with disease progression, the immune phenotype of MM cells is relatively conserved. For example, the malignant plasma cells in MGUS, SMM, and MM all express the key surface markers B cell maturation antigen (BCMA), CD38, and CD138. While BCMA expression significantly increases with disease progression, changes in CD38 and CD138 levels are less well-characterized (38, 39). In addition to these molecular features, MM cells rely on their BM microenvironment for growth, survival, and the development of therapy-resistant clones. Through cell-cell interactions and the secretion of cytokines, chemokines, and other factors, MM cells proliferate and impair the effector function of neighboring immune cells (38). For instance, in the BM of MM patients, key immunosuppressive cytokines are expressed at high levels (40, 41). These include interleukin-6 (IL-6), which mediates autocrine and paracrine growth of MM cells and inhibits tumor cell apoptosis, as well as TGF-β, which is an immune inhibitory factor that induces IL-6 secretion. Additionally, regulatory T cell (Treg) numbers are increased in MM patients, further suppressing the immune BM milieu (42, 43). The immunosuppressive characteristics of the molecular and cellular constituents of the BM microenvironment aid in disease progression and lead to poor clinical outcomes (37).

Given the immunosuppressive microenvironment of MM, successful therapies must simultaneously destroy malignant

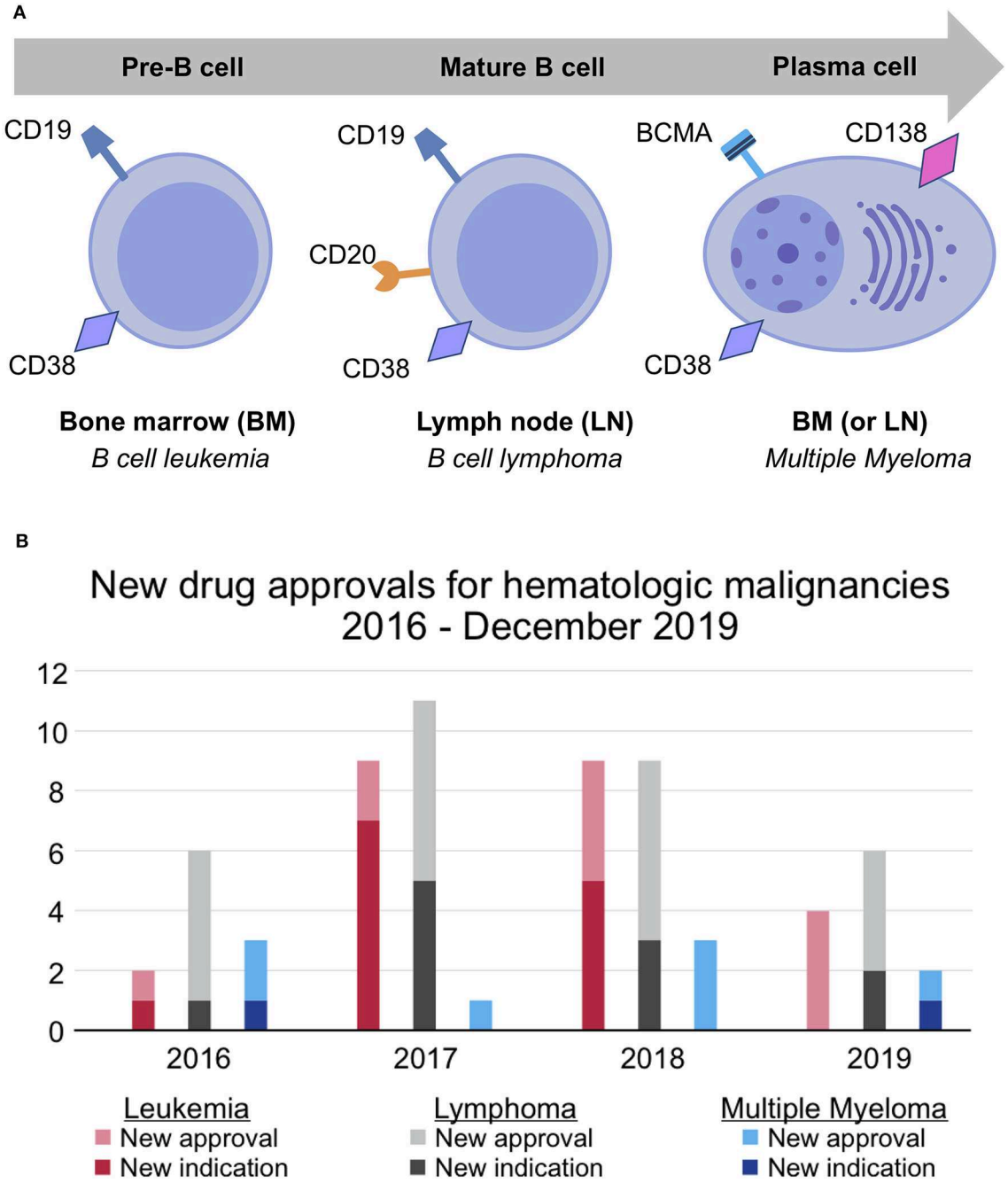

FIGURE 1 | Differences in molecular architecture and therapeutic success for hematologic malignancy subtypes. **(A)** B cell leukemia, B cell lymphoma, and multiple myeloma occur at different stages of the B cell lifecycle. Unlike pre- and mature B-cells, which express CD19 and CD20, plasma cells uniquely express BCMA and CD138. CD38 is expressed at all stages of the B cell lifecycle but is more highly expressed on malignant plasma cells. **(B)** New drug approvals for leukemia, lymphoma, and multiple myeloma between 2016 and December 2019 (21, 22). The pace of drug development for multiple myeloma has failed to keep pace with that of leukemia and lymphoma.

plasma cells and restore an effective anti-tumoral immune response (44). Such immunotherapies should (1) target surface molecules that are ideally expressed exclusively or at higher levels in MM cells than normal plasma or other immune cells and (2) bring effector immune cells into contact with MM cells, thereby enhancing effector cell-directed anti-tumor immunity. BsAbs meet these criteria, and therefore represent a next-generation immunotherapy with the potential to provide sustained clinical responses and even a cure for MM patients.

TABLE 1 | MM classification systems and definitions of high-risk disease.

Classification system	Features	Model	Year introduced	References
Durie-Salmon	Stage III One or more of the following: hemoglobin <8.5 g/dL; serum calcium >12 mg/dL; Advanced lytic bone lesions; high M-component production rates IgG value>7 g/dL, IgA >5 g/dL; urine light chain M-component >12 g/24 h	Tumor Burden/Stage	1975	Durie-Salmon Staging System (25)
International Staging System (ISS)	Stage III Serum β_2-microglobulin $\geq$ 5.5 mg/L (other stages consider serum albumin levels as well)	Tumor Burden/ Stage	2005	Greipp et al. (26)
University of Arkansas for Medical Sciences (UAMS) 17-gene model	High Risk Deregulated expression of 17 genes (1q32.1, 21q22.3, 1q21.2, 8q23.1, 10q23.31, 12q22, 1p36.21, 3p21.3, 7p14-p13, 1q22, 1q43, 1q31, 1p13.2, 1p22, 1p13.3, 2p22-p21, 6p21)	Cytogenetics	2007	Shaughnessy et al. (27)
Medical Research Council (MRC) Myeloma IX Trial	Adverse lesions defined as +1q21, del(17p13), del(13q14), or adverse IGH translocations t(4;14), t(14;16), or t(14;20) High Risk Presence of >1 adverse lesion Ultra-high Risk Presence of >1 adverse genetic lesions and ISS II or III	Combined Cytogenetics-ISS	2012	Boyd et al. (28)
mSMART	High Risk Genetic abnormalities on t(14;16), t(14;20), del(17p); GEP high risk signature	Cytogenetics	2013	Mikhael et al. (29)
International Myeloma Working Group (IMWG)	High Risk ISS II/III and t(4;14) or del(17p13)	Combined Cytogenetics-ISS	2014	Chng et al. (30)
Revised International Staging System (R-ISS)	Stage III ISS stage III (Serum β_2-microglobulin 5.5 mg/L) and either: high risk CA by iFISH (presence of del(17p) and/or translocation t(4;14) and/or translocation t(14;16), or high lactate dehydrogenase (LDH) (serum LDH > the upper limit of normal)	Combined Cytogenetics-ISS	2015	Palumbo et al. (7)
mSMART 3.0	High Risk Genetic abnormalities: t(4;14), t(14;16), t(14;20), del(17p), p53 mutation, +1q; RISS stage III; High plasma cell s-phase; high GEP risk signature	Combined Genetics-ISS	2018	Treatment Guidelines (31)

CA, cytogenetic abnormality; GEP, gene expression profiling; iFISH, interphase fluorescence in situ hybridization; IGH, immunoglobulin heavy; ISS, International Staging System; LDH, lactate dehydrogenase; mSMART, Mayo stratification of myeloma and risk-adapted therapy; R-ISS, Revised International Staging System.

BISPECIFIC ANTIBODIES: OVERVIEW, DESIGNS, AND POTENTIAL FOR MM

The idea of using BsAbs to redirect immune cells to tumor cells was first demonstrated in the 1980s and led to several clinical trials (45, 46). Catumaxomab (anti-EpCAM × anti-CD3) was the first BsAb to meet clinical approval by the European Union in 2009 (47). Blinatumomab (anti-CD19 × anti-CD3) was the first BsAb approved by the US Food and Drug Administration (FDA) in 2014 (48–50). Since then, one more BsAb—emicizumab, used to treat hemophilia A—has obtained FDA approval, and there are currently more than 60 BsAbs in various stages of preclinical and clinical development (51, 52). To date, no BsAb has been approved for use in MM patients, although there are 13 currently in clinical trials, and a pilot study evaluating the effects of blinatumomab in relapsed/refractory (R/R) MM patients was initiated in May 2017 (**Table 3**).

The majority of BsAbs are effector cell redirecting and most commonly involve αβ T cells via an anti-CD3 arm connected to a tumor antigen binding site. Anti-NKp30 BsAbs, which bind to natural killer (NK) cells, as well as BsAbs engaging CD16A and NKG2D, which bind to NK cells and γδ T cells, also exist in various stages of development (12, 15, 19). Two other types of BsAbs include tumor-targeted immunomodulators and dual immunomodulators. Tumor-targeted immunomodulators direct immune co-stimulation to pre-activated, tumor-infiltrating immune cells (e.g., tumor-specific effector T cells) by binding to a tumor antigen and a costimulatory molecule (e.g., 4-1BB on T cells). By activating a pool of many different tumor-specific T cell clones, rather than harnessing non-specific effector cells to one pre-determined tumor antigen, tumor-targeted immunomodulators may recognize tumor cells with antigen heterogeneity and build immunological memory. Dual immunomodulators bind two separate immunomodulating targets (usually T cell checkpoint pathways such as PD-1, LAG-3, or TIM-3) to block the mechanisms of the immunosuppressive tumor microenvironment (12). Importantly, BsAbs are effective in directing lysis of malignant cells with low antigen expression levels, a significant advantage when targeting surface molecules that are down-regulated as a mode of tumor evasion. Since no

TABLE 2 | Emerging high-risk MM factors.

Type	Factors	Year	References
Cytogenetic	t(14;16) (q32;q23); t(14;20) (q32;q23); Del(17p)	2016	Rajkumar (32)
Cytogenetic	FISH: t(4;14), t(14;16), t(14;20), del(17/17p), gain(1q); Non-hyperdiploid karyotype; Karyotype del(13); high-risk GEP70 signature	2016	Sonneveld et al. (33)
Cytogenetic	Primary translocations: t(4;14), t(14;16), t(14;20) Secondary translocations: MYC, jumping translocation 1q Copy change number: Isochromosome formation, hyperhaploidy, gain(1q), del(1p), del(17p) Homozygous inactivation of TSGs: Mutation +/- copy number change Genetic changes associated with DNA repair deficiency: genome-wide loss of heterozygosity	2017	Pawlyn and Morgan (34)
Epigenetic	Epigenetic modifier mutations; histone methylation and acetylation; DNA methylation, measured via mutations in DNA methylation modifiers, e.g., IDH1; microRNA	2017	Pawlyn and Morgan (34)
Bone Lesions	Presence of 3 large focal lesions, with a product of the perpendicular diameters > 5 cm^2	2018	Rasche et al. (35)

IDH1, isocitrate dehydrogenase 1; EZH2, enhancer-of-zeste 2 polycomb repressive complex 2 subunit; FISH, fluorescence in situ hybridization; GEP70, 70-gene expression profiling; MM, multiple myeloma; TSG, tumor suppressor gene.

TABLE 3 | Clinical trials of BsAbs targeting MM.

Targets	Drug name	Design	Trial type	Estimated enrollment	Estimated completion	References
BCMA × CD3	PF-06863135	IgG2a Fc region	Phase 1	80	Early 2022	NCT03269136
BCMA × CD3	TNB-383B	IgG4 Fc region	Phase 1	72	Late 2021	NCT03933735
BCMA × CD3	REGN5458	Fc region, Fab arms	Phase 1/2	56	Late 2022	NCT03761108
BCMA × CD3	REGN5459	Fc region, Fab arms	Phase 1/2	56	Late 2023	NCT04083534
BCMA × CD3	CC-93269	Trivalent, Fc region	Phase 1	19	Mid 2022	NCT03486067
BCMA × CD3	JNJ-64007957	IgG1 Fc region	Phase 1	120	Mid 2020	NCT03145181
BCMA × CD3	AMG420	BiTE	Phase 1	120	Early 2025	NCT02514239
BCMA × CD3	AMG701	Half-life extended BiTE (scFvs plus Fc region)	Phase 1	135	Mid 2025	NCT03287908
CD38 × CD3	AMG424	Fc region, scFv x Fab arms	Phase 1	20	Late 2022	NCT03445663
CD38 × CD3	GBR1342	Fc region, scFv x Fab arms	Phase 1	125	Early 2021	NCT03309111
CD19 × CD3	Blinatumomab	BiTE	Phase 1	20	Mid 2020	NCT03173430
FcRL5 × CD3	BFCR4350A	IgG1 Fc region	Phase 1	80	Mid 2021	NCT03275103
GPRC5D × CD3	JNJ-64407564	IgG1 Fc region	Phase 1	185	Mid 2021	NCT03399799

immunomodulatory BsAbs are currently in clinical trials for MM, this review will focus on immune cell redirecting BsAbs.

BsAbs can combine multiple functions of individual mAbs (such as direct cancer cell lysis, blocking malignant signaling pathways, independent T cell activation), entailing a comparatively simpler treatment regimen than one requiring the combination of multiple separate agents (12).

BsAbs are classed into ∼100 different formats, which fall roughly into two categories: BsAbs that include a fragment crystallizable (Fc) region, and BsAbs that consist of only fragment

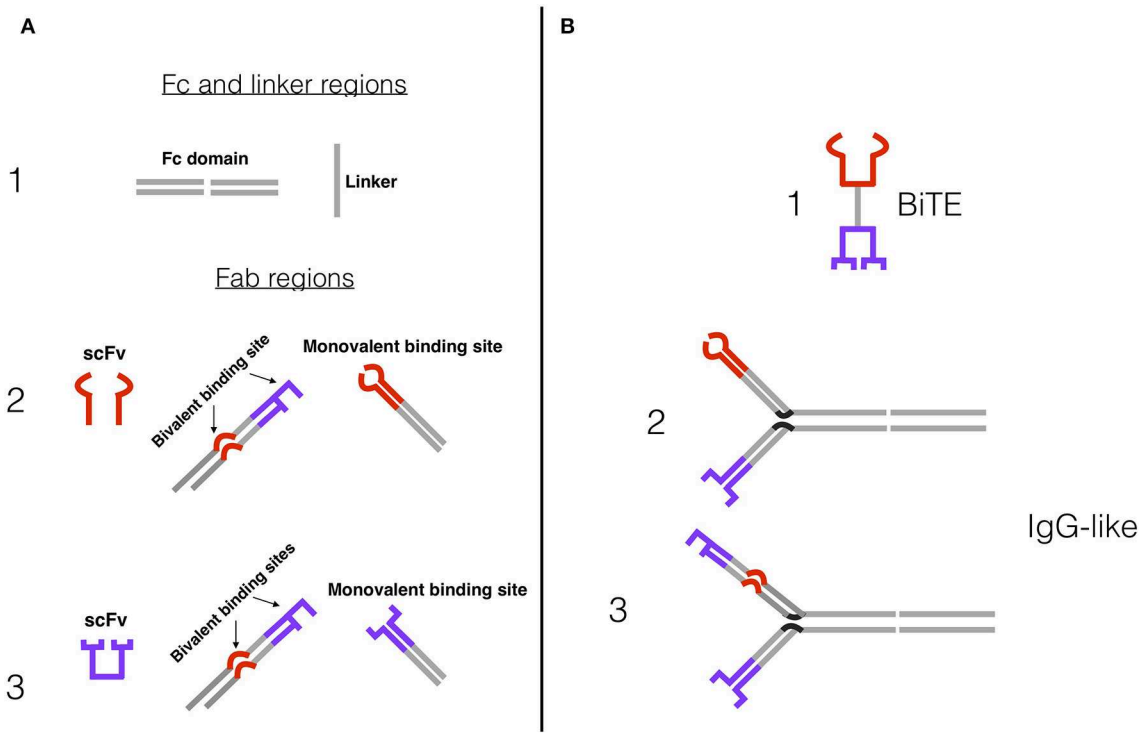

FIGURE 2 | Examples of BsAb constructs and designs. **(A)** (1) Fc domain and peptide linker, which connect antigen-binding sites in IgG-like BsAbs and Fc-less BsAbs, respectively. The Fc domain confers a number of additional features. (2) Effector cell binding sites, usually anti-CD3e for T cells or anti-CD16A for NK cells: single-chain fragment variable (scFv), bivalent binding site (tumor antigen and effector cell binding site), monovalent binding site. (3) Tumor antigen binding sites: scFv, bivalent binding site, monovalent binding site. **(B)** Simplified BsAb constructs. (1) Bispecific T cell Engager (BiTE) design, comprised of two scFvs and a peptide linker—e.g., blinatumomab. (2) Bivalent IgG-like BsAb design, with an Fc domain and two monovalent binding arms. (3) Trivalent IgG-like 2+1 BsAb design, with an Fc domain, a bivalent anti-tumor antigen and anti-CD3 or CD16A arm, and a monovalent anti-tumor antigen arm. Tetravalent BsAbs have a similar construction, but with two bivalent arms.

antigen-binding (Fab) variable regions and linkers. Both designs are being tested in MM. Some BsAbs with an Fc region (especially those that target NK cells rather than T cells) exhibit Fc-mediated effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC) via Fc receptor (FcR) binding, as well as added stability and a longer half-life (48, 53, 54). Anti-CD3 BsAbs often have an engineered, effector-silenced Fc region that mainly imparts a longer half-life and added stability (55). Fc-containing BsAbs are structurally similar to IgG molecules, with variations on the symmetry of their molecular composition and the number of binding sites (**Figure 2A**) (54). Increasing the number of binding sites on a BsAb (multivalency) can increase target affinity, especially for targets with low expression on tumor cells.

BsAbs that lack an Fc region merely consist of the antigen-binding sites of two antibodies, and most commonly follow the single-chain variable fragment (scFv) design. ScFvs only contain the variable regions of the heavy and light chains and are therefore the simplest iterations of the antigen binding site: They are relatively small and commonly connected by a peptide linker (56, 57). The scFv and linker format is utilized in the construction of bispecific T cell engager molecules (BiTEs), of which blinatumomab and AMG420 are examples (**Figure 2B**). The simplicity and small size of BiTEs and other scFv BsAbs confer both benefits (e.g., relative ease of adding additional scFvs to create trispecific or trivalent molecules) and drawbacks (e.g., short serum half-lives, decreased efficacy and increased cost by requiring repetitive dosing) (58, 59). Independent T cell activation (inducing cytotoxicity without requiring co-stimulation with CD28 or IL-2) has been observed in BsAbs with and without Fc region (12–19). Proposed mechanisms for this include clustering of the TCR-CD3 complexes to induce signaling in the absence of co-stimulation and the predominance of acting on memory T cells that require less stimulation to become activated (13). The wide array of BsAb structural designs and their advantages and disadvantages have been extensively reviewed by Brinkmann and Kontermann (54).

An alternative to exploiting the cytotoxic potential of T cells for tumor destruction is to redirect and activate NK cells. NK cell redirecting BsAb designs, such as Bispecific NK Engagers (BiKEs) (comprised of two scFvs) and BsAbs with modified Fc regions, are currently in clinical trials for hematologic malignancies; their use for MM is a promising future avenue (60, 61). Since NK cells are the first lymphocyte population to reappear after high-dose chemotherapy, NK cell redirecting BsAbs may be used to eradicate minimal residual disease (MRD) after first-line MM

treatment (62). Additionally, NK cell redirecting BsAbs have the potential to be used in combination with other treatments, such as adoptive NK cell transfer after autologous stem cell transplantation (autoSCT).

Their mechanism of action makes BsAbs unique candidates for high-risk MM therapy. High-risk MM patients often display a great degree of intra-tumoral genetic heterogeneity (63); therefore, activating the immune system for broad tumor recognition may be more promising than targeting single genetic lesions. The few studies that have investigated the treatment of patients with high-risk disease failed to conclude that intensification of personalized targeted therapy was significantly beneficial (64, 65). Even therapy regimens containing two autoSCTs ("tandem transplants") only delay disease progression in high-risk patients rather than cure it (66). The need for new therapies that effectively target high-risk and R/R MM is therefore great, and BsAbs have the potential to fulfill this need.

Several novel treatment approaches like chimeric antigen receptor (CAR) T cell therapies, targeted therapies, and combining mAbs are being implemented for high-risk and R/R MM patients, but BsAb therapies offer numerous advantages. Unlike CAR T cell therapies that have to be individualized by *ex vivo* manipulation of patient-derived T cells, BsAb therapies are "one size fits all" therapies that can be started immediately. BsAbs can be given in incremental doses and interrupted if necessary, so treatment-related toxicities are easier to manage than in CAR T cell therapies. This simplifies treatment regimens and study design/infrastructure and reduces costs (48, 67, 68). Notably, a recent report by Maruta et al. provides a direct comparison between target-reactivity and cross-reactivity of BsAb and CAR T cell models in MM, which showed similar tumor-killing activity, but a delay in CAR T cells relative to BsAbs (69). Additionally, targeted therapies directed at a particular genetic lesion (e.g., bortezomib, palbociclib, encorafenib, etc.) may only eradicate a certain subclone containing that lesion (e.g., the clone present in the diagnostic random iliac crest biopsy), whereas other clones (including disease-driving clones present in focal lesions) are spared (63). In contrast, BsAbs target antigens that are broadly expressed in all malignant plasma cells, such as BCMA, CD38, and CD138, and increase the chances of thoroughly eradicating all malignant clones. mAbs can similarly target tumor antigens, but are unable to directly harness the potent lytic power of T cells to aid in tumor destruction (70). The ADCC functions of mAbs are dependent on Fc functionality, which can be inhibited by alternative Fc glycosylation or Fc receptor polymorphisms, activation of inhibitory receptors, and competition with circulating IgG. BsAbs ensure effector cell involvement via their specific binding arm, guaranteeing the retargeting of effector cells against the malignant cell (47, 71). Thus, BsAbs present an unprecedented opportunity for all MM patients, and particularly those with high-risk and R/R MM for whom standard and targeted therapies have failed.

Despite the novel and promising features of BsAbs, these immunotherapeutics have faced considerable roadblocks on the path to commercial approval and widespread use. For T cell redirecting BsAbs, the activation of large proportions of non-specific T cells can lead to significant toxicity and treatment-related adverse events (AEs) (12). Cytokine release syndrome (CRS) is among the most important AEs of BsAb treatment, with multiple instances recorded in clinical trials of blinatumomab, PF-0683135, CC-93269, and AMG420 (68, 72–74). CRS can present as a variety of symptoms, ranging from influenza-like symptoms to neurotoxicity and multi-organ failure; the recommended treatment depends on its grade of severity (68, 75). Low-grade CRS can be treated symptomatically with antihistamines, antipyretics, and fluids, while high-grade CRS is treated with corticosteroids. Notably, a prophylactic protocol (consisting of dose adjustment and premedication with dexamethasone) for severe CRS was successfully devised to limit severe CRS during blinatumomab trials (68, 76). An additional study with dexamethasone and tocilizumab (anti-IL-6) has reduced CC-93269-induced CRS (77, 78).

NK cell redirecting BsAbs, which operate via FcR mediated cytotoxicity, present an alternative immunotherapy that may result in reduced general toxicity (12, 79, 80). However, to be successful in MM, NK cell redirecting BsAbs must find antigen epitopes that are not subject to competitive interference by serum IgGs (such as the high levels of M-paraprotein displayed in many MM patients) (79). CD16A, a type III FcγR, may be such an antigen (62). Hallmarks of tumor immune evasion, such as heterogenous expression and down-regulation of antigen levels, present obstacles to both T cell and NK cell redirecting BsAbs (79). New constructs, such as multivalent and tri-specific BsAbs, are under investigation as possible responses to these concerns (81–83). These new designs may also be pivotal in reducing toxicity.

MM DRUG TARGETS FOR BSABS

Ideally, BsAb therapeutic targets should be highly expressed on malignant cells and absent or at low levels on other cell types to avoid dose-limiting toxicities (84). Additionally, ideal BsAb targets play an important role in the survival and proliferation of malignant cells, preventing their down-regulation as a mechanism of tumor immune evasion (48). Antigen distribution and content vary both between patients and within a given patient, emphasizing that the success of each drug depends not only on construct but on target expression. So far, there are 24 BsAbs in development against eight MM targets (**Table 3**). Each MM target and its associated drugs will be discussed below, including ongoing clinical trials and preclinical developments.

BCMA (B Cell Maturation Antigen)

The most important MM drug target for BsAbs is BCMA (also known as TNFRSF17), which currently has eight BsAbs in clinical development (**Table 3**) and four in preclinical studies (**Table 4**). BCMA is a type III transmembrane glycoprotein belonging to the tumor necrosis factor receptor (TNFR) superfamily (90–93). BCMA is expressed primarily on B lineage cells and plays an important role in B cell proliferation (90). It is also widely expressed on all plasma cells, up-regulated during plasma cell differentiation, critical for long-term plasma cell survival, and overexpressed on malignant plasma cells and MM cell lines (90, 94–96) (**Figure 3**). BCMA is absent on most other cell types,

TABLE 4 | Preclinical models of BsAbs targeting MM.

Targets	Drug	Design	Model	References
BCMA × CD3	EM801	IgG1 Fc region	MM cell and effector cell co-cultures BMAs of MM patients (autologous T cells) NOG mice with human myeloma allogeneic xenograft Cynomolgus monkeys	Seckinger et al. (85)
BCMA × CD16A	AFM26	Tetravalent, Fc region	NK cell cultures, serum IgG MM cell and primary human NK cell co-cultures MM cell and PBMC co-cultures	Gantke et al. (62)
BCMA × NKp30	CTX-8573	IgG1 Fc region	MM and NK cell co-cultures, in the presence of sBCMA, sBAFF and sAPRIL Humanized mice models engrafted with MM tumors Cynomolgus monkeys	Watkins-Yoon et al. (15)
BCMA × CD3	AP163	Information not available	MM and effector cell co-cultures NSG mice models with human PBMCs and MM or Burkitt lymphoma tumor cells	Li et al. (16)
CD138 × CD3	STL001 or BiTE-hIgFc	scFvs and IgG1 Fc region	MM cell and PBMC co-cultures T cell activation assay NSG mice with human myeloma xenograft	Zou et al. (17)
CD138 × CD3	H-STL002 & M-STL002	scFvs and IgG1 Fc region	MM cell and PBMC co-cultures	Chen et al. (86)
CD38 × CD3	Sorrento CD38/CD3	scFv-Fc region fusion chain and Fab arm	MM cell and PBMC co-cultures NSG mice models with implanted MM or Burkitt lymphoma tumor cells and unstimulated PBMCs	He (87)
CD319 × NKG2D	CS1-NKG2D	BiKE	IL-2 primed NK cultures IL-2 primed PBMC with high, intermediate, low CS1 expression MM cell line co-cultures NSG mice engrafted with human PBMCs and high- and intermediate-CS1 expressing MM cell line xenografts	Chan et al. (19)
GPRC5D × CD3	GPRC5D TRAB	IgG region	MM cell and unstimulated PBMC co-cultures NSG mice model inoculated with human T cells and MM tumor cells NOG mice model engrafted with $CD34^+$ hematopoietic stem cells and MM tumor cells	Kodama et al. (88)
NY-ESO-1 × CD3	ImmTAC-NYE	TCR-like HLA-A2/NY-ESO-$1_{157-165}$arm, scFv, peptide linker	MM cell and $CD8^+$ cell co-cultures	McCormack et al. (89)
NY-ESO-1 × CD3	A2/NY-ESO-1_{157} –specific BsAb	anti-HLA-A2/NY-ESO-$1_{157-165}$ scFv, scFv, peptide linker	Peripheral blood T cells and T2 cells loaded with NY-ESO-1_{157} peptide co-cultures MM cell and peripheral blood T cell co-cultures Peripheral blood T cells and cells presenting the NY-ESO-$1_{157-165}$ peptide by HLA-A*02:06 co-cultures NOG mice model engrafted with MM cells and activated T cells	Maruta et al. (69)

except for low expression on plasmacytoid dendritic cells (pDCs) (90, 97). Importantly, and in contrast to other MM targets such as CD38, BCMA is not expressed on $CD34^+$ hematopoietic stem/progenitor cells (90).

BCMA has two cognate ligands: (1) B cell activating factor (BAFF, also known as TNFSF13B), which is necessary for B cell development and homeostasis, and (2) a proliferation-inducing ligand (APRIL or TNFSF13A). BAFF and APRIL, either as membranous ligands or in the cleaved, soluble form, bind to BCMA to promote plasma cell growth and survival. Upon ligation by BAFF or APRIL, BCMA activates downstream signaling pathways including the NF-κB, ETS-1 like protein 1 (Elk-1), JNK, ERK, and MAPK pathways (**Figure 3**) (98–101). This induces pronounced up-regulation of the MCL-1 and BCL-2 anti-apoptotic proteins, preventing dexamethasone-induced cell death. MM patients have up to five times higher soluble BAFF and APRIL serum levels than healthy individuals (102). BCMA also associates with three known TNFR-associated factors (TRAFs)—TRAF1, TRAF2, and TRAF3—which are signal transducers that bind to several members of the TNFR superfamily and facilitate activation of NF-κB, Elk-1, and JNK signaling pathways (101).

Membranous (m)BCMA expression levels per cell increase as healthy plasma cells transform from normal into malignant cells through the disease progression of MGUS to MM (90, 103). Similarly, soluble (s)BCMA levels increase with disease progression, and are found at increased serum levels in MM patients (104). sBCMA levels are also inversely proportional to overall and progression free survival rates (105). sBCMA, which is released from the membrane by spontaneous γ-secretase

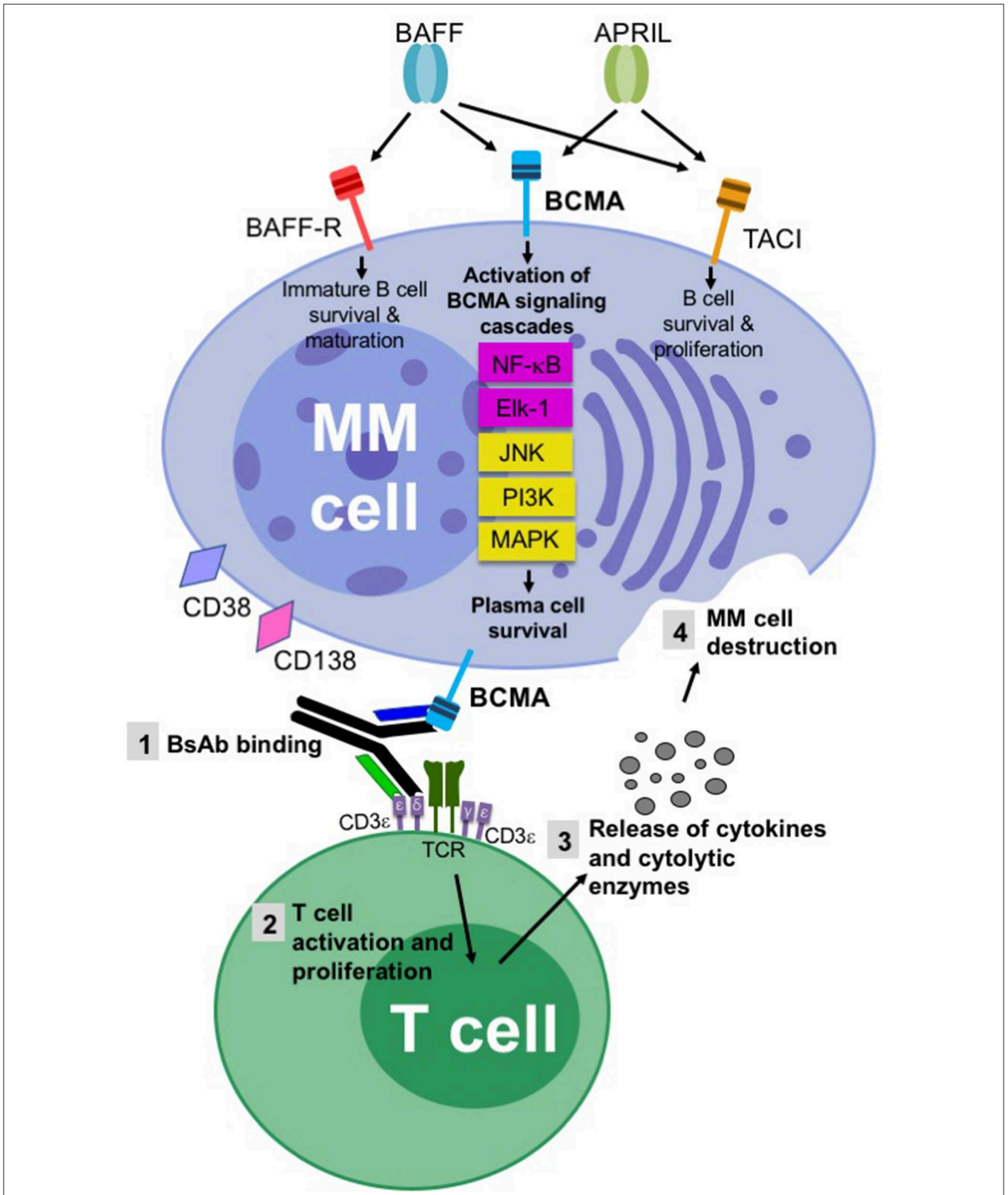

FIGURE 3 | Schematic of key tumor targets and the mechanism of action of BsAbs in multiple myeloma. The superior aspect of the figure highlights the importance of the BCMA/BAFF/APRIL axis and the associated BCMA signaling pathways for malignant plasma cell survival. The inferior aspect of the figure provides a schematic of how BsAbs induce effective T cell-directed MM cell death. A T cell redirecting BsAb binds to BCMA on a MM cell and CD3e on a T cell, coupling these two cells.

(Continued)

FIGURE 3 | NK cell redirecting BsAbs bind to CD16A rather than CD3e. Alternative BsAb targets on MM cells include CD38, CD138, FcLR5, CD19, CD319, GPRC5D, and NY-ESO-1. TCR-CD3e cross-linking leads to the activation and proliferation of $CD4^+$ and $CD8^+$ T cells. Cytokines (i.e., IFN-g, IL-2, IL-6, TNF-a) and cytolytic enzymes (i.e., granzyme B and perforin) are released, resulting in MM cell death.

activity, negatively regulates mBCMA signaling and its associated pathways by competing with mBCMA for BAFF and APRIL (105, 106). Studies suggest that the sequestering of BAFF by sBCMA prevents BAFF from binding to mBCMA and BAFF-R on both healthy and cancer cells. In MM, this blocks the stimulation of normal antibody production, thus contributing to the immunosuppressive MM phenotype by inducing hypogammaglobulinemia (105). Additional studies have shown that high levels of sBCMA may weaken the effectiveness of anti-BCMA type therapies, including BsAbs (104, 107). Additionally, the accumulation of sBCMA in the BM may inhibit BsAb recognition of tumor cells, and reduced mBCMA levels on malignant cells (due to their release as sBCMA by γ-secretase) may further facilitate tumor evasion. These effects may be mitigated by γ-secretase inhibitors (104, 107).

MM patients also display elevated BCMA expression levels on pDCs, which are often in close proximity to MM cells in the BM, and are present in higher numbers in MM patients than in healthy controls (97). pDCs were shown to promote MM progression by secreting factors that enhance MM cell growth (i.e., IL-6), MM chemotaxis (i.e., CXCL-12), and BM angiogenesis (i.e., VEGF), and induce local immunosuppression (i.e., IL-10) (90, 97, 108). Furthermore, pDCs can be resistant to MM therapies, such as bortezomib, lenalidomide, and dexamethasone (97). Given the expression of BCMA on pDCs and their role in MM progression, BCMA-targeting BsAbs have the potential to co-eradicate a key pro-tumorigenic immune cell subset in the BM microenvironment.

Importantly, BCMA seems to be of relatively limited importance to other cell types and tissues. BCMA is not involved in early B cell development or B cell homeostasis, which is in contrast to BAFF receptor (BAFF-R) and Transmembrane Activator and CAML (calcium modulating cyclophilin ligand) Interactor (TACI), which are key to these processes (**Figure 3**) (102, 109). BCMA-deficient mice develop normally and display healthy physical appearances (110), and only the survival of long-lived BM plasma cells is impaired when compared to wild-type mice (90, 94). In contrast to BCMA, the importance of which to MM is clearly documented, BAFF-R is absent on malignant plasma cells and TACI is expressed at lower levels compared to BCMA (103). Because BCMA's crucial functions in the maintenance and survival of MM cells make its down-regulation unlikely, the likelihood of tumor evasion and drug resistance during treatment is low (85). Collectively, BCMA's plasma cell-specific expression pattern, its overexpression on MM cells and its active involvement in the malignant phenotype make it an ideal BsAb therapeutic target.

Clinical Trials of BCMA-Targeting BsAbs

PF-06863135 (PF-3135)

PF-06863135 is an anti-BCMA x anti-CD3 BsAb that consists of targeting arms within an IgG2a Fc backbone. It has a half-life of 4–6 days in cynomolgus monkeys (111). PF-06863135 is currently under investigation in a dose-escalation phase 1 trial (ClinicalTrials.gov identifier NCT03269136). The study population includes adult patients with R/R MM, who previously received a proteasome inhibitor, an immunomodulatory drug, and/or an anti-CD38 mAb. Patients received escalating doses of PF-06863135 intravenously once a week to determine the maximum tolerated dose (MTD) and recommended phase 2 dose (RP2D). Results from 23 patients treated weekly over a median duration of 4 weeks showed one complete response, two minimal responses, and nine stable disease cases. Every patient developed more than one AE; most events were grade 1–2, with 5 patients developing grade 3 events. CRS was the most common treatment-related AE, affecting six patients. CRS primarily occurred after the first dose, was dose-dependent and resolved in all patients in less than 4 days. Dose escalation is ongoing as of early 2020, with plans to continue until the maximum tolerated dose is reached (73).

TNB-383B

TNB-383B is a trivalent anti-BCMA x anti-CD3 BsAb, with a bivalent anti-BCMA arm (112). The BsAb has a silenced human IgG4 Fc region, with a 10-day half-life in cynomolgus monkeys (113). Preclinical studies testing the drug in BM samples from seven R/R MM patients showed that TNB-383B induced MM cell death, dose-dependent T cell activation, and less cytokine secretion than other BsAbs. NOD/SCID/IL-2Rγ-deficient (NSG) xenograft mouse models showed that TNB-383B reduced tumor growth *in vivo* (114). In June 2019, a phase 1 dose-escalation and expansion trial (NCT03933735) of TNB-383B in patients with R/R MM, who have received at least 3 prior lines of therapy, was initiated. Study arm A will investigate escalating doses of single-agent TNB-383B (25 μg to 40 mg per dose) once every 3 weeks, and arm B will involve an expansion cohort after the recommended dose is established. As of 2019, 12 patients have been enrolled in arm A, with no grade 3 or higher treatment-related AEs (115).

REGN5458

REGN5458 is an anti-BCMA x anti-CD3 BsAb, with an Fc domain and anti-BCMA/anti-CD3 Fab domains (116). In preclinical studies, REGN5458 increased surface levels of BCMA on MM cell lines, in addition to inducing T cell killing of MM cells and cytotoxicity in primary human plasma cells (117). In NSG mice, REGN5458 inhibited xenografted tumor growth at doses of 0.4 mg/kg, and at ten times lower doses in immunocompetent mouse models (117). In cynomolgus monkey studies, REGN5458 depleted $BCMA^+$ plasma cells in the BM (117). The REGN5458 treatment induced a mild inflammatory response in the cynomolgus monkeys characterized by transiently increased C-reactive protein and serum cytokines, but was otherwise well-tolerated (117). In a comparative study with CAR T cells, REGN5458 displayed targeted cytotoxicity of MM cells lines and primary plasmablasts.

Additionally, administration of REGN5458 to NSG mice led to clearance of systemic OPM-2 myeloma tumors within 4 days (compared with CAR T cells, which required 10–14 days for tumor clearance) (117). A clinical trial (NCT03761108) of a first-in-human study of the drug was initiated in January 2019, in patients with R/R MM. The study involves cohorts of multiple REGN5845 dose levels administered intravenously. Results from seven patients after 4 weeks of treatment showed four responses, of which two were MRD negative. Three responders have ongoing responses after a duration of follow-up ranging from 1 to 5.2 months. Every patient had at least one treatment-related AE, five of which were grade 3 or higher (116).

REGN5459

REGN5459 is an anti-BCMA x anti-CD3 CD3 BsAb, with an Fc domain and with different binding characteristics from REGN5458 (118). A first-in-human clinical trial (NCT04083534) was initiated in September 2019, in patients with R/R MM. The study involves cohorts of multiple REG5845 dose levels administered intravenously and is expected to end in 2023.

CC-93269 (formerly EM901)

CC-93269 is an anti-BCMA × anti-CD3 trivalent BsAb with a bivalent anti-BCMA arm for increased avidity and an IgG1 based Fc region (85, 119, 120). A phase 1 trial (NCT03486067) of a dose-escalation and expansion study of the drug in patients with R/R MM started in April 2018, consisting of intravenous infusion on 28-day cycles (78). As of October 2019, 30 patients had received the drug, with doses ranging from 0.15 to 10 mg. Preliminary results suggest that higher doses ($\geq$3 mg) of CC-93269 result in improved clinical outcomes: overall response rates were 36% in patients treated with 3–6 mg and 89% in patients treated with >6 mg. None of the patients receiving <3 mg responded. The complete response rate was 17% overall, and 44% among the 9 patients treated with 10 mg. The median response rate was 4.1 weeks (range 4.0–13.1), and 92% of responders achieved MRD negativity, often by the end of the first cycle. During follow-up, 29 of the 30 patients experienced at least one treatment-related AE, and 22 patients (73%) experienced a grade 3 or higher AE. The most common treatment-related AEs were neutropenia (43%), anemia (37%), infections (30%), and thrombocytopenia (17%). Twenty-three patients (77%) developed any-grade CRS, including one grade 5 (i.e., death). Most CRS events were successfully managed using dexamethasone and tocilizumab (78).

JNJ-64007957

JNJ-64007957 is an anti-BCMA × anti-CD3 BsAb with an IgG1 Fc region (121, 122). In preclinical pharmacokinetic and tolerability studies conducted on cynomolgus monkeys, JNJ-64007957 was well-tolerated at doses up to 10 mg/kg per week. No toxicologically significant effects were found when administered once a week for a 5-week period. The pharmacokinetic report suggested a low anti-drug antibody response, indicating that this drug can be safely administered multiple times per week (123). A phase 1 trial (NCT03145181) of a dose-escalation and expansion study of JNJ-64007957 in patients with R/R MM started in May 2017. The study is being conducted in two parts: one for intravenous administration and one for subcutaneous administration. Additionally, a phase 1 trial (NCT04108195) of subcutaneous daratumumab in combination with intravenous JNJ-64007957 or JNJ-64407564 (i.e., an anti-GPRC5D BsAb) in patients with MM started in January 2020. This study is being conducted in two parts, beginning with a dose escalation phase consisting of 28-day cycles, followed by a dose expansion part. It is expected to end in 2020.

AMG420 (formerly BI 836909)

AMG420 is an anti-BCMA × anti-CD3 human BiTE antibody comprised of two scFvs (124). In preclinical studies with co-cultures of unstimulated peripheral blood mononuclear cells (PBMCs) and MM cell lines, AMG420 induced redirected lysis of MM cells and target-dependent release of cytokines by T cells. Anti-tumor activity was further examined in two NSG mouse models reconstituted with human T cells and either subcutaneous or intravenous MM cell line xenotransplantations (125). Comparable dose-dependent anti-tumoral activity was observed in both subcutaneous and intravenous administration regimens of AMG420. Toxicity studies in cynomolgus monkeys showed a dose-dependent decrease of healthy plasma cells in the BM (126). A phase 1 first-in-human dose-escalation and expansion study (NCT02514239) of the drug in patients with R/R MM started in July 2015. Results from this study, which enrolled 42 patients, showed 13 responses, including 6 MRD-negative complete responses, 3 complete responses, and 4 partial responses (127). The median response time was 1 month, and 11 patients responded within the first treatment cycle. Of the 7 patients dosed at 400 μg/d, 5 had complete response with no presence of MRD, and 2 had partial responses. No major toxicities were observed. Thus, 400 μg/d was set as the MTD (128). Of the 42 patients enrolled in this trial, 7 discontinued treatment due to AEs, of which 6 were considered serious, including CRS (3 instances), peripheral polyneuropathy (1 instance), edema (1 instance), and pyrexia (1 instance) (128, 129). Nineteen patients (45%) experienced SAEs, of which infection (14 instances) was most commonly reported (74, 127).

AMG701

AMG701 is an anti-BCMA × anti-CD3 human BiTE comprised of two scFvs and an Fc region for extended half-life (∼5 days in non-human primates) (14, 130). Preclinical studies of AMG701 in MM cell lines and patient samples showed significant induction of T cell-mediated lysis of MM cells, even at low concentrations and low effector:target cell (E:T) ratios (2:1 and 1:2). This finding was also confirmed in drug-resistant MM cell lines or in the presence of MM-supporting osteoclasts. AMG701 also induced lysis of tumor cells from R/R MM patients in tumor and effector cell co-cultures. Analysis of AMG701-treated MM and effector cell co-cultures revealed that AMG701 induced $CD8^+$ and $CD4^+$ T cell proliferation (47.5 and 16.7% at 10 ng/ml, respectively) and T cell activation (up-regulation of CD25 and CD69). AMG701 also increased the differentiation of naive $CD4^+$ and $CD8^+$ T cells toward the central and effector memory phenotype. Additionally, it was postulated that the

proliferating T cells potently lysed even those MM cells with reduced BCMA expression. In xenograft MM mouse models, AMG701 blocked tumor growth after 5 days and completely eradicated growth after three injections at all dose levels (0.02, 0.2, and 2 mg/kg); however, with AMG701 treatment alone, mice experienced tumor regrowth by the end of the study. Mice treated with a combination of AMG701 and the immunomodulator and anti-MM agent lenalidomide experienced significant anti-tumor activity 2 days after their first injection, and their tumor volume remained low even after 45+ days of the study. In *in vitro* MM cell and effector cell co-cultures, the AMG701 lenalidomide combination treatment enhanced MM cell-killing as compared to AMG701 treatment alone, including in the presence of MM-supporting cells from the BM microenvironment [such as osteoclasts and bone marrow stromal cells (14, 131)]. A phase 1 trial (NCT03287908) of a dose-escalation and expansion study of the drug in patients with R/R MM started in November 2017 and is expected to end in 2026.

Preclinical Models of BCMA-Targeting BsAbs

EM801

EM801 is an anti-BCMA × anti-CD3 trivalent BsAb with a bivalent anti-BCMA arm for increased avidity and an IgG1 based Fc region (85). Preclinical studies in human MM cell lines showed that EM801 binds T cells and MM cells, leading to TCR/CD3 cross-linking and activating the CD3 downstream signaling pathways. In BM aspirates from MM patients, EM801 induced significant primary myeloma cell death by autologous T cells, reaching 90% reduction in 48 h (85). To further evaluate the anti-tumor activity of EM801, studies were performed in human MM xenografted immunodeficient mice and in cynomolgus monkeys. In mice, EM801 was potent against highly proliferating MM cells. In monkeys, a reduction in $BCMA^+$ plasma cells was observed in the BM and peripheral blood (85).

AFM26

AFM26 is an anti-BCMA × anti-CD16A tetravalent BsAb, consisting of a bivalent anti-CD16A arm for increased NK cell avidity connected to a bivalent anti-BCMA arm by an IgG-like backbone (62, 79). AFM26 binds to an epitope of CD16A that is not blocked by serum IgG (e.g., M-protein) binding. A preclinical study conducted on NK cell cultures found that AFM26 exhibits prolonged retention on the surface of NK cells, with receptor retention levels of AFM26 remaining above 60% after 1 h, even in the presence of serum IgG. This is particularly significant for MM because high M-protein levels are characteristic of the disease. In the same preclinical study, AFM26 was applied to primary human NK and MM cell co-cultures (E:T, 5:1), and induced MM cell-specific lysis. In an experiment conducted on multiple MM cell lines with varying BCMA expression levels, AFM26 was found to retain potency even on cells with low BCMA expression. Additionally, unlike mAbs such as daratumumab and elotuzumab (anti-SLAMF7), AFM26 did not induce NK cell lysis. Furthermore, when compared with BiTE incubation on MM and PBMC co-cultures, AFM26 displayed comparable efficacy and markedly reduced cytokine production, suggesting a superior safety profile (62, 132).

CTX-8573

CTX-8573 is an anti-BCMA × anti-NKp30 BsAb, with an IgG1-like afucosylated Fc region to additionally engage CD16A on NK cells and γδ T cells (15). It displays a 16-day half-life in cynomolgus monkeys (133). CTX-8573 was tested on human NK and MM cell co-cultures and displayed potent cytotoxicity toward $BCMA^+$ tumor cells, including on low BCMA-expressing cell lines. Significantly, this cytotoxic activity was maintained in the presence of sBCMA, sBAFF, and sAPRIL, all of which are displayed at higher levels in high-risk MM patients. A model of the BsAb with an aglycosylated Fc region (to preclude CD16A engagement) retained ADCC, showing that NKp30 engagement alone can promote innate cell activation and cytotoxicity, although CD16A involvement enhances such activity. A preclinical study conducted on multiple humanized mouse models engrafted with MM tumors showed potent anti-tumor activity. Pharmacokinetic and safety profiling in cynomolgus monkeys showed standard biphasic pharmacokinetics and no evidence of systemic immune activation as measured by C-reactive protein levels (133).

AP163

AP163 is an anti-BCMA × anti-CD3 BsAb with a 9 h half-life in cynomolgus monkeys (16). Preclinical testing on MM cell lines and effector cell co-cultures showed that AP163 induces cross-linking, T cell activation, cytokine production, proliferation, and redirected target cell killing, eradicating tumor cells. AP163 was studied in multiple NSG mice models injected with human PBMCs and subcutaneous MM cell xenografts or BCMA-expressing Burkitt's lymphoma cell xenografts. In all models, AP163 resulted in T cell activation, cytokine production, and cancer cell killing. In the two MM xenografts tested, AP163 eradicated or significantly delayed tumor growth at doses as low as 0.04 mg/kg. Toxicity testing was carried out on cynomolgus monkeys and non-human primates, in which the drug was well-tolerated at doses up to 5 mg/kg. Significantly, AP163 induced minimal cytokine release as compared to conventional BsAbs (16).

CD138 (Syndecan-1)

CD138 is a type I transmembrane protein of the syndecan proteoglycan family (134). CD138 has a wide variety of functions, including cell signaling, cell-cell adhesion, cytoskeletal organization, and tumorigenesis (i.e., proliferation, angiogenesis, and metastasis) (135). It is expressed primarily on epithelial cells, transiently on developing mesenchymal cells and at the terminal plasmacytic differentiation stage of B cells (136, 137). Viable MM cells have high expression of membranous (m)CD138; when cells undergo apoptosis, shedding of mCD138 is triggered (138, 139). Studies have found that CD138 suppresses apoptosis in MM cells by activating the insulin-like growth factor-1 receptor; high mCD138 expression can thus indicate non-apoptotic cells, making it an efficient antigen for targeting viable MM cells (140, 141). CD138 also acts as a co-receptor for TACI and APRIL, promoting the APRIL/TACI-associated pathways that induce survival and proliferation of MM cells (142). Additionally, soluble (s)CD138, which is proteolytically shed by matrix

metalloproteases and sheddases, is present at high levels in the serum of MM patients and is heavily implicated in disease progression: it acts as a key mediator between MM cells and the BM microenvironment on which they rely, promoting signaling pathways that lead to tumor cell proliferation, angiogenesis, and metastasis. As such, sCD138 is an independent predictor of poor prognosis in MM (143–145).

Various anti-CD138 mAb and T cell engaging MM therapies have taken advantage of the high expression levels of CD138 on MM cells. One mechanism involves coating tumor cells with anti-CD138 mAbs as a method of enhancing dendritic cell cross-presentation of the tumor antigen and the generation of myeloma specific killer T cells (146). CD138's elevated expression on MM cells and its active role in the disease phenotype make it a promising MM BsAb target. Additionally, mCD138's role in preventing apoptosis likely makes tumor cells addicted to this molecule, although a significant proportion of patients were shown to have CD138-negative MM clones (141, 147). Potential drawbacks of CD138 include its high expression on epithelial cells and the accumulation of sCD138 in the BM. In a first-in-human phase 1 trial of an anti-CD138 DM4 (a derivative of the cytotoxic agent maytansine)-antibody conjugate in R/R MM patients, patients suffered from common epithelial-related AEs (e.g., hand-foot syndrome, xerophthalmia, stomatitis, and blurred vision) (148). Furthermore, the characteristic, accelerated shedding of sCD138 and its accumulation in the BM of MM patients may inhibit BsAb recognition of tumor cells. As of February 2020, CD138-targeting MM BsAbs have not yet entered clinical trials.

Preclinical Models of CD138-Targeting BsAbs

STL001 (also known as BiTE-hIgFc)

STL001 is an anti-CD138 × anti-CD3 BsAb with two scFv arms and an IgG1 Fc region to allow for FcR-mediated NK binding. A preclinical study tested the effects of STL001 on PBMC and MM cell co-cultures (E:T 7:1) and compared the cytotoxicity to that of an anti-CD138 mAb and an anti-CD3 mAb combination and various controls (17). STL001 induced lysis of 90.1% of MM cells after 48 h, compared to 70.5% in the mAb combination and 13.8% and 12.3% in the controls. STL001 was also incubated in a T cell activation assay consisting of PBMCs from healthy donors and IL-2, whereby T cell activation was measured by CD25 and CD69 expression levels. After 24 h, STL001 showed 78.12–85.45% T cell activation efficiency. After 2 weeks of PBMC stimulation and activation, STL001 bound over 96% of the total NK cells. STL001 was also tested at an intravenous dose of 3 mg/kg in an NSG xenograft MM tumor mouse model that had also been injected with unstimulated healthy human PBMCs (E:T, 3:1). Compared to the isotype control, STL001 significantly impaired MM tumor growth, resulting in an ∼75% decrease in the mean tumor volume relative to the control (17).

H-STL002 and M-STL002

H-STL002 and M-STL002 are anti-CD138 × anti-CD3 BsAbs with two scFv arms and an IgG1 Fc region. A preclinical study tested the effects of these BsAbs on PBMC and MM cell co-cultures (86). After 20 h of incubation, 74–80% of T cells were activated (measured by CD69 expression), and significant MM cell lysis was observed at E:T ratios as low as 7:1. Furthermore, cytotoxicity activity of 98.4% and 98.3% was measured for M-STL002 and H-STL002, respectively (86).

CD38 (Cyclic ADP Ribose Hydrolase)

CD38 is a type II glycoprotein of the ADP-ribosyl cyclase family, with ectoenzymatic and receptor functionality (149, 150). CD38 plays a regulatory role in calcium homeostasis, nicotinamide adenine dinucleotide (NAD) signaling, and weak adhesion events (151). Originally thought to be a lymphocyte-specific antigen, CD38 was shown to be expressed in nearly every type of tissue, but with elevated expression on hematopoietic cells (151). CD38 is expressed at varying stages of B cell development (i.e., in BM precursor B cells and in terminally differentiated plasma cells) and serves as a marker of T lymphocyte development (149). Additionally, CD38 is uniformly and highly expressed in MM, making it attractive for BsAb targeting. In its role as a receptor, CD38 binds to CD31 (PECAM-1), which is expressed on endothelial cells, lymphoid cells and in the lungs and kidney (152). Interactions between CD38 and CD31 regulate adhesion events between $CD38^{+}$ cells and human umbilical vein endothelial cells. These interactions are also involved in the binding and migration of leukocytes through the endothelial wall, the activation and proliferation of leukocytes, and in B cell development (152, 153). The role of CD38-CD31 interactions is important for MM cell survival in the BM by mediating adhesion to BM endothelial and stromal cells. Clinical studies examining mAb agents that target CD38—such as daratumumab—often lead to down-regulation of CD38 surface expression. Although down-regulation of a target antigen is usually undesirable, in this case it may be beneficial, leading to reduced interaction and support of MM cells by the BM microenvironment (154, 155). A potential obstacle to CD38's use as an MM target is its expression on T cells; however, a preclinical study has shown that T cell fratricide does not preclude the efficacy of anti-CD38 BsAbs as long as tumor cells are lysed at a higher or equal rate to T cells (18).

Clinical Trials of CD38-Targeting BsAbs

AMG424

AMG424 is an anti-CD38 × anti-CD3 BsAb, with an Fc domain, an anti-CD38 scFv, and an anti-CD3 Fab domain (18). In a preclinical study using MM target cells co-cultured with purified human T cells (E:T 10:1), AMG424 induced complete target cell lysis and limited cytokine release, compared to other BsAbs with higher CD3 affinities. In human PBMC and MM cell line co-cultures (E:T 1:1), AMG424 triggered a pronounced depletion of MM cells and normal B cells, induced a 2-fold increase in T cell numbers and triggered robust T cell activation as measured by induction of CD25. Likewise, in cynomolgus monkeys, intravenous injection of AMG424 triggered T cell activation. However, it also triggered depletion of T cells, B cells, lymphocytes, and monocytes. In an orthotopic tumor model in NSG mice reconstituted with human T cells, intravenous injection of AMG424 induced tumor regression and T cell activation (18). While AMG424 also depleted T cell numbers,

the E:T ratio remained stable. A phase 1 first-in-human trial (NCT03445663) of the drug in patients with R/R MM started in July 2018. Part 1 of the study aims to assess the safety and tolerability of AMG424 and determine the MTD and/or biologically active dose. Part 2 will further evaluate the safety and tolerability of the MTD. The trial is expected to end in 2022.

GBR1342

GBR1342 is an anti-CD38 × anti-CD3 BsAb, with an Fc domain, an anti-CD38 scFv and an anti-CD3 Fab domain; it has a half-life of ∼5 days in rats (156, 157). In human PBMC and MM cell co-cultures (E:T 10:1), GBR1342 demonstrated potent killing of MM cells (157). Additionally, in redirected lysis assays, it demonstrated greater potency than commercial anti-CD38 antibodies, such as daratumumab (157). A phase 1 first-in-human dose-escalation and expansion study (NCT03309111) in patients with previously treated MM began in 2017, with GBR1342 administered by intravenous infusion at an initial dose of 1 ng/kg, with varying dose escalations by cohort (up to 1,000 ng/kg) in 28 day cycles (157). Part 1 of the study is dose evaluating and aimed to assess the safety and tolerability of GBR1342. Part 2 will focus on efficacy exploration. Preliminary results from 19 patients revealed 28 treatment-related AEs in 14 patients, two of which were treatment-related and reversible (i.e., a creatine phosphokinase elevation and an infusion-related reaction, with no neurotoxicity observed to date). Of the 19 patients, 4 were still undergoing treatment with GBR1342 in 2018; as of 2020, the longest duration on the drug has been five cycles, with one patient entering his sixth cycle of dosing at 400 ng/kg (158). In September 2019, GBR1342 was granted orphan drug designation by the FDA (159).

Preclinical Models of CD38-Targeting BsAbs

Sorrento anti-CD38/CD3 BsAb

Sorrento Therapeutics' anti-CD38/CD3 BsAb has an anti-CD38 Fab arm and an anti-CD3 scFv-Fc region fusion chain. The fusion chain has hinge mutations for reduced Fc region affinity/effector function, to decrease antigen-independent T cell toxicity (87). A preclinical study showed that the BsAb induced potent lysing of $CD38^+$ MM cell lines, with antigen density positively correlating with cytotoxic potency. In an *in vivo* follow up, the BsAb construct with the most prolonged anti-tumor activity and best T cell stimulation was the one with a balanced CD38 and CD3 affinity. In a cytotoxicity assay using human PBMCs and MM cell lines, the BsAb showed more potent tumor cell killing than the daratumumab control. In NSG mice models with implanted CD38-expressing Burkitt's lymphoma tumor cells and previously unstimulated human PBMCs, BsAb treatment inhibited tumor growth and prolonged survival. An investigational new drug application is projected to be filed for the BsAb in the first half of 2020 (160).

CD19

CD19 is a type I transmembrane glycoprotein member of the immunoglobulin superfamily (161). CD19 is primarily involved in the immune response, by modulating B cell receptor (BCR)-dependent and independent signaling to establish B cell signaling thresholds (162–164). It works as the lead receptor in a complex with CD21, CD81, and CD225 to decrease the threshold for receptor-dependent signaling, acting as a co-receptor for BCR signal transduction and interacting with various down-stream protein kinases (including the Src family, Ras family, Abl, Bruton's tyrosine kinase, adapter molecules, and PI3K) (161, 165–169). CD19 is expressed on B cells, from the pre-B cell stage and throughout development, with expression decreasing during terminal plasma cell differentiation (161, 162, 168). CD19 expression is further reduced as plasma cells transform into MM cells. It has been proposed that CD19 loss aids MM cell proliferation (170), and this loss precludes MM patients from benefitting from anti-CD19 therapies. However, there have been reports of R/R MM patients responding to anti-CD19 CAR T cell therapies in combination with other treatments (171, 172). Additionally, super-resolution microscopy has shown very low CD19 expression on MM cells, which was undetectable by flow cytometry but may be accessible to antibodies and modified effector cells (173). These findings make CD19 a potentially interesting BsAb target for MM, despite its unconventional expression pattern and unclear role in the disease phenotype.

Clinical Trials of CD19-Targeting BsAbs

Blinatumomab

Blinatumomab is an anti-CD19 × anti-CD3 BiTE made of two scFvs, with a half-life of ∼2 h in humans (174). In July 2017, it was approved by the FDA for treatment of R/R B cell precursor acute lymphoblastic leukemia (B-ALL) in adults and children (175). Importantly, blinatumomab is the first FDA-approved BsAb. In the phase III trial, which confirmed the clinical benefit of blinatumomab in B-ALL, the drug increased median survival rate from 4 to 7.7 months and resulted in a higher rate of event-free survival than chemotherapy (31% vs. 12%) (TOWER trial, NCT02013167) (176). SAEs including neurologic events, CRS, administration-site reactions, and procedural complications, were reported in 62% of patients treated with blinatumomab as compared to 45% in the chemotherapy group. Results from a phase II study (BLAST, NCT01207388) evaluating blinatumomab in B-ALL found a median OS of 36.5 months after treatment, and more than 50% of patients who achieved MRD after their first cycle were alive at 5 years (72).

Given the success of blinatumomab in B-ALL, it is currently in clinical trials for numerous other B cell malignancies, including R/R MM (177). A phase 1 clinical trial of blinatumomab in combination with salvage autoSCT for patients with R/R MM began in May 2017 (NCT03173430). The study consists of administering up to two 28-day cycles of blinatumomab to patients who previously received high-dose melphalan and autoSCT for MM, with results pending. A case study of blinatumomab-induced response of R/R MM in the context of a secondary pre-B cell ALL emerged in 2017 (172). The patient, a 70-year-old female, developed pre-B-ALL while undergoing lenalidomide therapy for MM, for which she was in partial remission. She underwent cytoreductive therapy and began blinatumomab induction, which resulted in a complete remission of her ALL and a very good partial response of her MM by International Myeloma Working Group criteria (172). Although

this case is promising for the application of blinatumomab to other MM patients, it is important to point out that this patient's MM cells stained positive for CD19, which is atypical for MM tumors (172).

CD319 (SLAMF7 or CS1)

CD319 is a homophilic (self-ligand) surface glycoprotein receptor of the signaling lymphocyte activation molecule (SLAM) family (178). CD319 is a regulatory receptor, with a key role in immune cell function and immune signaling mediation (178, 179). CD319's cytoplasmic tail includes an immunoreceptor tyrosine switch-motif (180). The immunoreceptor tyrosine switch-motif mediates binding to Ewing sarcoma/Friend leukemia integration 1 transcription factor-activated transcript 2 (EAT-2), a member of the SLAM-associated protein (SAP) family of adaptors. CD319/EAT-2 binding determines whether CD319 stimulation will activate or inhibit immune cell functions; in the presence of EAT-2, CD319 plays an activating role, while in the absence of EAT-2, it mediates inhibitory effects (178). CD319 is expressed predominately on NK cells, but also $CD8^+$ T cells and B cells, with marked up-regulation during terminal B cell differentiation into plasmablasts and plasma cells (181). It is absent on hematopoietic stem/progenitor cells and blood cancers, except for malignant plasma cells (182, 183). CD319 mRNA has been detected on over 97% of $CD138^+$ MM cells, with protein expression confirmed by flow cytometry (184). The function of CD319 in plasma cells and MM cells is not certain: both seemingly lack EAT-2, theoretically suggesting an inhibitory role for CD319 mediation, as is the case in EAT-2-negative NK cells (178, 185). However, a study testing isolated and activated B cells found that stimulation with anti-CD319 mAbs (along with an anti-CD40 mAb and IL-4) increased cell proliferation and induced the expression of growth-supporting cytokines (179). This suggests the possibility of an activating role for CD319 on MM cells, despite their lack of EAT-2. CD319 may also aid in the communication and adhesion between MM cells and the BM microenvironment. A study investigating the effects of an anti-CD319 mAb found that CD319 is localized to the uropod membrane domains of MM cells, regions promoting cell-cell adhesion (183). When CD319 was blocked by the mAb, MM cell adhesion to BM stromal cells was reduced in a dose-dependent manner (183). By supporting adhesion of MM and BM stromal cells, CD319 may promote MM cell proliferation and survival. The ubiquitous and elevated expression of CD319 on MM cells and its possible involvement in disease progression make it a promising BsAb target. As of February 2020, CD319-targeting MM BsAbs have not yet entered clinical trials.

Preclinical Models of CD319-Targeting BsAbs

CS1-NKG2D BsAb

CS1-NKG2D BsAb is an anti-CD319 × anti-NKG2D bispecific T/NK cell engager made of two scFvs (19). NKG2D is expressed on cytolytic immune cells such as NK cells, $CD8^+$ T cells, γδ T cells, and NKT cells (with no expression on $CD34^+$ hematopoietic stem/progenitor cells). It is one of the major activating NK cell receptors and a co-stimulatory molecule on cytotoxic $CD8^+$ T cells and NKT cells (186). A preclinical study incubated IL-2-primed NK cell cultures with NKG2D and found that the CS1-NKG2D BsAb binds to and triggers the activation of NK cells (19). The study then tested the effects of the BsAb on three different co-cultures consisting of IL-2-primed PBMCs with MM cell lines with high, intermediate, and low CD319 expression. Dose-dependent increases in MM cell lysis were observed in the high and intermediate expression co-cultures. The BsAb was then tested in primary MM patient peripheral blood samples treated with allogeneic PBMCs (E:T, 10:1), which reduced MM cells with high CD319 expression. No specific lysis against T, NKT, or NK cells was found. NSG mice engrafted with human PBMCs and CD319 high- and intermediate-expressing MM cell lines were also administered subcutaneous doses of the BsAb. In this context, only mice engrafted with CD319 high-expressing MM cell lines experienced a significant prolonged survival in response to the BsAb (i.e., ∼40 days compared to ∼30 days in the control group) (19).

FcRL5 (Fc Receptor-Like 5)

FcRL5 (CD307) is a membrane protein that is closely related to the Fc receptor family. FcRL5 regulates BCR signaling and binds aggregated IgG (84, 187). It is restricted to B lineage cells, with high expression on mature B cells and plasma cells (187). FcRL5 mRNA is overexpressed in MM cells, and one study found FcRL5 protein expression to be three times higher on MGUS and MM cells than on normal plasma cells (84). Another study found comparable expression levels between normal and malignant plasma cells, but higher expression on plasma cells than on normal B cells (188). Significantly, the FcRL5 gene is located at the chromosomal breakpoint in 1q21, the amplification of which is associated with aggressive MM (189). A study analyzing primary MM biopsies found a significant correlation between FcRL5 mRNA expression and 1q21 gain, suggesting that the 1q21 gain can lead to FcRL5 overexpression in high-risk MM patients (188, 190). Therefore, the development of FcRL5-targeting BsAbs may be especially valuable for high-risk MM patients. One concern about FcRL5 as a BsAb target is its large extra-cellular region: large antigen size (in particular large extracellular regions) and increased distance from the epitope to the target cell membrane can interfere with efficient T cell synapse formation (191). However, constructing an anti-FcRL5 BsAb that targets an epitope on the most membrane-proximal domain of FcRL5's extracellular region is an effective solution to this issue; such a BsAb has displayed promising preclinical results at picomolar concentrations (188).

Clinical Trials of FcRL5-Targeting BsAbs

BFCR4350A (formerly RO7187797)

BFCR4350A is an anti-FcRL5 × anti-CD3 BsAb with an IgG1 Fc region (188, 192). BFCR4350A's anti-FcRL5 arm is constructed to bind to an epitope chosen for its location on the most membrane-proximal extracellular domain, and its ability to achieve efficient synapse formation. In preclinical studies, BFCR4350A was applied to MM cell and $CD8^+$ or $CD4^+$ T cell co-cultures, resulting in dose-dependent T cell activation and killing of the MM cells. It also induced robust T cell proliferation, with 95% of the $CD8^+$ T cells undergoing up to six

cell divisions in 5 days (188). BFCR4350A was then tested on co-cultures of patient-derived BM mononuclear cells (BMMCs) with healthy-donor $CD8^+$ T cells and healthy BMMCs. BFCR4350A displayed similarly cytotoxic dose-dependent killing of myeloma BMMCs and of normal plasma cells. The preclinical study also examined the activity of BFCR4350A in humanized NSG mice with transplanted $CD34^+$-purified human hematopoietic stem cells. The mice were subcutaneously inoculated with MM cells and later given weekly IV doses of 0.5 mg/kg of BFCR4350A, which resulted in tumor regression in all mice. A study consisting of a single intravenous dose with slow infusion of 1–4 mg/kg of BFCR4350A was conducted in cynomolgus monkeys (188). The treatment resulted in T cell activation, transient T cell decrease, complete depletion of B cells in the spleen and BM, robust dose-dependent depletion of B cells in the lymph nodes, a dose-dependent reduction of IgG levels, and mild cytokine release (188). Collectively, plasma cell and IgG depletion suggest effective BFCR4350A activity in the BM. A second preclinical study, testing the efficacy of single host cell construction of BFCR4350A (i.e., *in vivo* as opposed to *in vitro* assembly), found comparable results between these construction methods (193). A phase 1 dose escalation and expansion trial (NCT03275103) of the drug in patients with R/R MM started in September 2017 (**Table 3**). The drug is being administered intravenously in 21-day cycles, up to a maximum of 17 cycles or unacceptable toxicity, and the expected primary completion date is 2021.

GPRC5D (G Protein-Coupled Receptor Class C Group 5 Member D)

GPRC5D is a transmembrane orphan receptor of the G protein-coupled receptor family, whose functions are poorly characterized (194–196). MM patients have high *GPRC5D* mRNA expression in their BM, with low expression in normal tissues (194). GPRC5D is also highly expressed on the surface of MM cells, with lower expression on B and plasma cells and no expression on other hematopoietic cells (88). Due to this expression pattern, GPRC5D is thought to play a key role in MM tumor cell proliferation (197). *GPRC5D* mRNA expression has also been associated with the high-risk cytogenetic events del(13q14) and t(4;14), suggesting its possible role as a prognostic marker (194). Therefore, GPRC5D is an interesting and novel target for MM.

Clinical Trials of GPRC5D-Targeting BsAbs

JNJ-64407564

JNJ-64407564 is an anti-GPRC5D × anti-CD3 BsAb with an IgG1 Fc region (198, 199). A preclinical study tested JNJ-64407564 in a co-culture of MM cell lines and healthy human T cells (E:T, 5:1), a co-culture of healthy human whole blood and MM cell lines, and a co-culture of BMMCs from MM patients and exogenous healthy human T cells (200). JNJ-64407564 induced MM cell directed cytotoxicity in all co-cultures and dose-dependent T cell proliferation in the MM cell line and healthy human T cell co-culture. T cell activation was observed in both healthy human T cell co-cultures but not in the blood co-culture. JNJ-64407564 was then tested in two NSG mice models with human MM xenografts and human PBMCs; the drug led to significant anti-tumor activity and 100% complete responses in both groups. Testing of the drug in cynomolgus monkeys showed no adverse effects (200). A phase 1 dose-escalation and expansion trial (NCT03399799) in patients with R/R MM began in 2017, with JNJ-64407564 administered by intravenous or subcutaneous injection. Additionally, a phase 1 trial (NCT04108195) testing combinations of daratumumab with JNJ-64407564 (anti-GPRC5D BsAb) or JNJ-64007957 (anti-BCMA BsAb) in MM patients started in January 2020. This study is being conducted in two parts, beginning with a dose escalation phase consisting of 28-day cycles, followed by a dose expansion part, and is expected to end in 2021.

Preclinical Models of GPRC5D-Targeting BsAbs

GPRC5D TRAB

GPRC5D TRAB (T-cell redirecting antibody) is an anti-GPRC5D × anti-CD3 BsAb with an IgG base. A preclinical study testing four prototypes examined their anti-tumor activity (88). Two prototypes were added to a co-culture of unstimulated human PBMCs and GPRC5D-expressing MM cell lines and to a control of unstimulated human PBMCs and GPRC5D-negative lung cancer cell lines, respectively. Both prototypes induced cytotoxicity against the MM cells but not the lung cancer cells; GPRC5D expression levels on MM cells did not strongly impact cytotoxicity. The effects of two BsAbs were also tested in an NSG mouse model xenografted with human T cells and GPRC5D-expressing MM cell lines and in a NOG mouse model engrafted with human $CD34^+$ hematopoietic stem/progenitor cells and xenotransplanted with a MM cell line.

Importantly, these mouse models used MM cell lines that possess t(4;14), a translocation associated with high-risk MM (88, 201). In the NSG model, treatment with 10 mg/kg of the BsAb prototypes led to significant reduction in volume of both tumors as compared to the non-tumor specific control BsAb, curing up to 50% of the mice. In the NOG model, IV treatment with 10 mg/kg of the BsAb prototypes induced tumor regression in 60% of mice. The cytotoxicity of GPRC5D TRAB against these MM models suggest that this molecule may represent a promising treatment candidate for high-risk MM patients.

NY-ESO-1 (New York Esophageal Squamous Cell Carcinoma 1)

NY-ESO-1 (also known as cancer/testis antigen 1B, CTAG1B) is an immunogenic member of the cancer/testis antigen family—a protein family with germ and cancer cell expression patterns—showing nuclear localization in mesenchymal stem cells and predominately cytoplasmic expression in tumor cells (202, 203). Little is known about the biological function of NY-ESO-1, but its structural features and expression patterns have suggested a role in cell cycle progression and growth, apoptosis, germ cell self-renewal and differentiation, and stem and cancer cell proliferation (203–207). Its expression in healthy tissue is limited to testis and placental cells, but it is expressed in a wide range of tumor types, including MM. NY-ESO-1 expression is particularly high in relapsed patients and patients with cytogenetic abnormalities as defined by gene expression

profiling (208). In 335 newly diagnosed MM patients, NY-ESO-1 expression was present in 60% of cases in patients with cytogenetic abnormalities vs. 31% of cases with no abnormalities; this number increased to 100% and 61% at relapse, respectively (208). These findings suggest that NY-ESO-1 expression may correlate with MM clonal evolution.

NY-ESO-1 is highly immunogenic, with the ability to elicit simultaneous humoral and cellular immune responses (208, 209). In MM, antibody responses to NY-ESO-1 have been found to correlate with tumor load and disease progression (208, 210). NY-ESO-1-derived peptides are presented on MHC class I molecules, allowing for T cell recognition (69). Spontaneous $CD8^+$ T cell responses (recognizing NY-ESO-1 peptides 157–165 presented on HLA-A2) have been exhibited in MM patients, and laboratory expansion of these T cells has resulted in efficient MM cell killing (208). The HLA-A2/NY-ESO-$1_{157-165}$ peptide complex is therefore being used as a target antigen in the development of NY-ESO-1-targeting BsAbs for MM (69, 89). The tumor-specific expression of NY-ESO-1 and its elevated prevalence in high-risk patients makes it a promising BsAb target; however, its MHC machinery-dependent presentation may result in loss of expression as a means of immune escape. The combination of HLA-A2/NY-ESO-$1_{157-165}$-targeting BsAb therapy with agents that increase the expression of MHC-machinery proteins, such as interferon (IFN)-γ, may be an avenue worth exploring, to reduce the chances of immune escape via HLA-A2 down regulation (211–214). As of February 2020, NYE-ESO-1-targeting MM BsAbs have not yet entered clinical trials.

Preclinical Models of NY-ESO-1-Targeting BsAbs

ImmTAC-NYE

ImmTAC-NYE (immune-mobilizing monoclonal TCR against cancer) is an anti-NY-ESO-1 × anti-CD3 BsAb, consisting of a TCR-like, anti-HLA-A2/NY-ESO-$1_{157-165}$ arm fused to an anti-CD3 scFv arm via a peptide linker (89, 215). A preclinical study of the BsAb in a co-culture of MM cells and $CD8^+$ effector T cells (E:T, 10:1) showed dose-dependent tumor lysis at 0.1–10 nM (89). ImmTAC-NYE was able to bind to cells with a low-density of HLA-A2/peptide complexes, suggesting maintained functionality despite MHC down-regulation. The study also found that ImmTAC-NYE-activated T cells release cytokines IFN-γ, IL-2, and TNF-α, which, in addition to attracting effector immune cells to the tumor site, may spur long-term anti-tumor activity by promoting components of the death receptor pathway in tumor cells, providing an additional mechanism of tumor cell killing even after the BsAb is metabolized (89, 216).

A2/NY-ESO-1_{157}-specific BsAb

A2/NY-ESO-1_{157}-specific BsAb is an anti-NY-ESO-1 × anti-CD3 BsAb consisting of an anti-HLA-A2/NY-ESO-$1_{157-165}$ scFv connected to an anti-CD3 scFv via a peptide linker (69). A preclinical study testing the BsAb on a co-culture of peripheral blood T cells and antigen presentation-deficient T2 cells loaded with NY-ESO-1_{157} peptide showed that the BsAb triggered T cell production of IFN-γ, IL-2, and TNF-α. The BsAb only released cytokines in the presence of the NY-ESO-1_{157}-loaded cells and did not appear to be activated by CD3 binding alone, indicating reduced general toxicity. The BsAb was also tested in a peripheral blood T cell and MM cell co-culture, where it triggered cytokine production and killing of MM cells. A NOG mouse model engrafted with MM cells and activated T cells showed that 10-μg doses of the BsAb significantly suppressed tumor growth. The cross-reactivity of the BsAb with different HLA-A2 alleles was then tested by incubating it in peripheral blood T cell co-cultures with cells presenting the NY-ESO-$1_{157-165}$ peptide by HLA-A*02:06 instead of HLA-A*02:01. Levels of reactivity between the two different alleles were comparable, suggesting that the BsAb would be successful in patients with either type of HLA-A2. The study, which also directly compared the BsAb to a CAR T cell construct with the same anti-HLA-A2/NY-ESO-$1_{157-165}$ scFv, found that the anti-tumor effects of the BsAb were seen earlier than those of the CAR T cell therapy. This may be explained by the fact that the cytolytic synapses induced by the BsAb were more similar to those formed by TCR binding to HLA/peptide complexes than the synapses induced by the CAR T cells were (69).

FUTURE DIRECTIONS AND CONCLUSIONS

The development of BsAb treatments for MM has great potential. Preclinical findings in *in vitro* and *in vivo* models have shown effective tumor eradication. Additionally, preliminary clinical results of BCMA-targeting BsAbs PF-06863135 and AMG420 have been promising, with an absence of dose-limiting toxicity in PF-06863135 and six MRD-negative complete responses in AMG420 (73, 74). As more study results materialize, BsAbs will continue to be refined to increase efficacy and safety.

Multiple areas of further development are already emerging, with a focus on reducing treatment-related adverse events and on conquering tumor evasion.

Down-regulation of the target antigen is a classic mechanism of tumor resistance, and multivalent BsAb constructs that increase target avidity, as well as trispecific antibodies that target more than one tumor antigen, may be methods to overcome this obstacle (82). Targeting multiple tumor antigens in one antibody may also prove useful in addressing the heterogeneity of target expression on malignant cells. The increased specificity provided by trispecific antibodies may lead to new combinations of MM targets or to novel targets altogether.

Multi-target specificity may also be a crucial development for avoiding B cell aplasia, leukopenia, and the accompanying increased risk of infection, by ensuring that only the cells that express a particular antigen combination are directly targeted, reducing the chance that healthy cells are lysed. Because many target antigens are also expressed on non-transformed B cells and plasma cells (and are thus targeted by effector T cells), the depletion of B and plasma cell compartments poses a risk of AEs like infection. Febrile neutropenia occurs in up to 40% of B-ALL patients treated with blinatumomab (48, 217). In most patients receiving higher doses of blinatumomab, hypogammaglobulinemia has been observed, but there has been no evidence for an increase in long-term infectious

complications. A study examining the long-term effects of CD20-expressing B cell depletion in lymphoma and rheumatoid arthritis patients undergoing rituximab therapy was conducted in 2011. It found that multiple courses of drug exposure may result in IgG and IgM levels below the lower limit of normal serum levels, halted plasma cell formation, and higher serious infection risks (218). B cell aplasia resulting from CAR T cell therapy has been addressed by intravenous or subcutaneous immunoglobulin replacement therapy, and it has been suggested that the same response can be used during BsAb treatment until B cells have recovered (48, 219). Emerging BsAb technologies may prevent against unnecessary B and plasma cell depletion by increasing their specificity. A promising example of such technology is the "split" trispecific antibody, which is divided into two scFv halves, both connected to the same anti-CD3 antibody (83). The CD3-binding site only becomes functional when *both* scFvs have attached to their target antigen, ensuring that effector cell lysis is only directed at cells expressing both antigens. Thus, antigen combinations uniquely expressed by the cancer cells can be targeted, without accompanying B cell and plasma cell depletion (83).

Multi-target specificity is likely to also reduce BsAbs' toxicity profile by limiting the instances of T cell activation. Increasing target avidity may be another way to decrease unwanted cell lysis and the associated risks of aplasia and cytotoxicity. Asymmetric BsAb constructs with bivalent sites for the tumor antigen may not only increase the strength of binding to tumor cells, but also avoid CD3 activation in the absence of sufficient target antigens (e.g., in the case of low antigen expression on healthy cells) (81).

Independent T cell activation, a feature displayed by some but not all BsAbs, is an important area of further development. In some MM cases, inhibition of co-stimulation in the tumor microenvironment via expression of co-inhibitory molecules aids tumor evasion by neutralizing T cell activity (82). BsAb designs that induce biological synergies, resulting in independent T cell activation without the need for costimulatory molecules (e.g., the designs of anti-BCMA BsAb AMG420, anti-CD38 BsAb AMG424, and anti-FcRL5 BsAb BFCR4350A), are thus important for further development (18, 125, 188). Trispecific antibody models designed to stimulate two T cell antigens (rather than only CD3) aim to increase and prolong T cell activation without the need of external co-stimulatory models. One such MM-specific model targets CD38 on the tumor cell, and stimulates both CD3 and CD28 on the T cell (220). CD28, the most important "second signal" on T cells, is also expressed on MM cells at low levels (221, 222). A preclinical study testing the effects of the trispecific antibody was conducted on co-cultures of human PBMCs and MM cell lines and in NSG mouse models xenografted with human $CD8^+$ T cells and MM cell lines. The study found that the inclusion of the CD28-binding site not only eliminated the need for external co-stimulation, but also prolonged T cell survival, improved recognition of MM cells, reduced non-specific toxicity, and contributed significantly to anti-tumor efficacy. In the NSG mouse model, tumor growth was completely suppressed in the presence of antibody doses as low as 1 μg/kg (220). These results encourage the further development of trispecific anti-CD28 arm-including antibodies and are particularly promising for MM antibodies, given their increased specificity resulting from MM cell CD28 expression.

Combining BsAbs with immune checkpoint inhibitors (ICIs) may play a key role in the advancement of MM-targeting BsAb therapy by preventing against T cell exhaustion. T cell exhaustion is a feature of MM that may be exacerbated by treatment with BsAbs (223, 224). PD-1/PD-L1 signaling is a hallmark of tumor immunosuppression and T cell exhaustion. Increased PD-1 and PD-L1 expression has been observed in MM patients throughout the course of disease progression, resulting in T cell deactivation and allowing for tumor growth (225). Clinical cases of BsAb-induced T cell exhaustion have been recorded, with a blinatumomab-resistant ALL patient displaying an increase of PD-L1-expressing B-precursor ALL cells (224). Significantly, preclinical findings in MM-targeting BsAbs have also suggested induced T cell exhaustion: increased PD-1 expression in T cells after stimulation by anti-FcRH5/CD3 BsAb in the presence of target-expressing MM cells was observed in cynomolgus monkeys and led to reduced lysis of PD-L1 expressing target-cells (188). However, such mechanisms of T cell exhaustion can be therapeutically countered using ICIs (81). Combinations of anti-PD-1/PD-L1 mAbs with BsAbs have enhanced T cell activation and proliferation and increased cancer cell lysis *in vitro* in multiple studies (226, 227). In MM, the addition of an anti-PD-L1 mAb to anti-FcRH5/CD3 BsAb therapy significantly increased the efficiency of MM cell killing *in vitro* and *in vivo*, restoring T cell activity (188). Such combinations of ICIs with BsAbs may be pivotal in developing treatments that are responsive to immunosuppression.

Combinations of BsAbs with therapeutic Treg depletion may also assist in fighting against immunosuppression. A potential concern about BsAb treatment is that independent T cell activation may also activate unwanted Tregs (82). In MM, Treg numbers are abnormally high, aiding immunosuppression of effector cells in the BM microenvironment (42). Combinations of BsAbs with therapeutic Treg depletion may be helpful or necessary. A preclinical study examining Treg levels in the blood of 42 blinatumomab-treated R/R B-ALL patients confirmed a negative correlation between Treg levels and response to blinatumomab therapy. Importantly, depleting Tregs *in vitro* restored the blinatumomab-triggered proliferation activity of patient T cells. Therapeutic Treg depletion may be achieved *in vivo* by treating patients with cyclophosphamide or fludarabine before blinatumomab therapy (228). Co-infusion of ICIs and manipulation of BsAb design to recruit additional cell types may also prove valuable in overcoming immunosuppression (82). An interesting model of BsAb design ingenuity can be seen in "TriKEs," which are trispecific killer cell engagers. TriKEs are like NK cell redirecting BsAbs (e.g., BiKEs), with the added integration of IL-15 to drive expansion of NK cells for increased anti-tumoral activity. Preclinical studies in AML have shown TriKE activity to be more efficient than BiKE activity (229). Similarly, innovative BsAb designs such as BsAb-armed T cells are also being applied to MM (230). A clinical study (NCT00938626) targeting myeloma precursor cells in standard and high-risk MM patients administered the patients with anti-CD3 × anti-CD2 BsAb-armed activated T cell infusions prior

to autoSCT (230). The infusions induced anti-myeloma IFN-γ and anti-SOX-2 IgG responses, which were then boosted in every patient post-autoSCT. Such responses have been shown to be associated with reduced risk of progression from MGUS to MM (231). This finding suggests that BsAb-armed activated T cell infusions can induce cellular and humoral anti-myeloma immunity that can be detected and boosted after autoSCT (230).

Bispecific immunoconjugates, which consist of two tumor-targeting arms linked to a cytotoxic agent, are another frontier of BsAb innovation with the potential to benefit MM patients. A BsAb-cytokine conjugate—20-C2-2b, which targets tumor antigens CD20 and HLA-DR and is fused to two copies of IFN-α2b—has shown potent inhibition of MM cell lines. A preclinical study found that this compound showed potent cytotoxicity against MM cell lines, even those with limited expression of CD20 or HLA-DR individually (232). As the BsAb field continues to grow and the clinical data accumulates, ongoing innovations will be implemented to improve the immunotherapeutic options for patients with MM and numerous other cancers.

Despite recent therapeutic advances, existing treatments remain largely ineffective for high-risk and R/R MM. Novel immunotherapies, especially BsAbs, provide a new treatment approach for these patients. With numerous phase 1 clinical trials of MM-targeting BsAbs currently underway, the prospect of this new immunotherapeutic treatment for MM patients is on the horizon.

AUTHOR CONTRIBUTIONS

CS conceived of the review. CC drafted the manuscript with help from SK. CC and DP created the figures. CC created the tables. DP, CC, and CS edited the manuscript. CC and CS revised the manuscript, and all authors approved its final version.

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Anti-CS1 × Anti-CD3 Bispecific Antibody (BiAb)-Armed Anti-CD3 Activated T Cells (CS1-BATs) Kill CS1$^+$ Myeloma Cells and Release Type-1 Cytokines

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Background: Multiple myeloma (MM) remains incurable despite *significant* advances in chemotherapy, targeted therapies, and immunotherapy. Bispecific antibody (BiAb)-armed activated T cells (BATs) have been developed for targeting and treatment of solid and hematologic malignancies. BATs are serial killers of tumor cells, secrete Th_1 cytokines, and induce adaptive cellular and humoral immune responses in patients (pts). This study provides preclinical data using bispecific anti-CS1 (elotuzumab) × anti-CD3 (OKT3) antibody (CS1Bi)-armed activated T cells (CS1- BATs) that provide a strong rationale for applying CS1-BATs to pts with MM.

Methods: CS1-BATs and unarmed activated T cells (ATC) were incubated with MM cell targets at various effector to target ratios (E:T) in a quantitative flow cytometry-based assay to determine the degree of cell loss relative to target cells incubated without ATC. ATC from up to 8 normal donors were armed with various concentrations of CS1 BiAb and tested against 5 myeloma cells lines for CS1-BATs-mediated killing and release of Th_1 cytokines, chemokines and granzyme B.

Results: CS1-BATs from normal donors killed each of 5 MM cell lines proportional to E:T ratios ranging between 1:1 and 10:1 and arming concentrations of 12.5 to 50 ng/million ATC, which was accompanied by release of Th_1 cytokines, chemokines and granzyme B. CS1-BATs prepared from MM pts' peripheral blood mononuclear cells (PBMC) showed increasing cytotoxicity and T cell expansion over time against ARH77 MM cells. The optimal arming dose of CS1Bi is 50 ng/10^6 ATC.

Conclusions: These data demonstrate the therapeutic potential of CS1-BATs-mediated cytotoxicity and Th_1 cytokines release at low E:T and support advancing their clinical development in pts with MM.

Keywords: bispecific antibody, elotuzumab, activated T cells, OKT3, multiple myeloma, cytotoxicity, cytokines, chemokines

INTRODUCTION

Multiple myeloma (MM) is the second most common hematologic malignancy. Patients are the most sensitive and responsive to the first line of therapy, which provides the highest chance of achieving minimal residual disease (MRD) negativity. With subsequent lines of therapy, the depth and duration of response typically lessens and many patients ultimately become refractory to treatment. With the introduction of proteasome inhibitors (PIs), immunomodulatory agents (IMiDs), histone deacetylase (HDAC) inhibitors and monoclonal antibodies (mAb), the number of patients achieving 5 year survival in 2019 is now over 50% (seer.cancer.gov). Despite the effectiveness of combination therapies, autologous stem cell transplant (autoSCT) and maintenance, MM remains an incurable disease. Non-toxic specific anti-MM approaches that induce long-term anti-MM immunity are needed to purge residual CD34-CD138- clonogenic cells from the marrow to improve progression-free survival (PFS) and overall survival (OS). The goal of therapy is to achieve the deepest possible response with MRD negativity since the probability of long-term remission is highest in MRD negative patients (1). Signaling lymphocytic activation molecule family 7 (SLAMF7) is a cell surface receptor, also called cell-surface glycoprotein CD2 subset 1 (CS1), expressed at high levels on MM cells and at lower levels on NK cells where it acts as an activating receptor. The overexpression of CS1 in MM in more than 90% of cases, irrespective of cytogenetic abnormalities (2), makes it an attractive target for immunotherapy. Elotuzumab (Elo) is a humanized immunoglobulin G1 immunostimulatory antibody targeted against CS1. It works by activating natural killer cells, mediating antibody-dependent cell-mediated cytotoxicity (ADCC), and may further enhance cytotoxicity by promoting CS1-CS1 interactions between NK cells and CS1+ target cells independent of ADCC (3). Interestingly, Elo does not directly mediate anti-MM activity as a single agent, but works synergistically with IMiDs (4).

Our strategy combines the cellular cytotoxicity of ATC with the anti-CS1 targeting specificity of Elo. OKT3, which is directed at the activating CD3-epsilon chain of the T cell receptor (TCR), is chemically heteroconjugated to anti-CS1 to form CS1Bi. Arming of *ex vivo* expanded ATC with CS1Bi converts each ATC into an anti-CS1 cytotoxic T lymphocyte (CTL). Although we have reported preclinical work, as well as clinical trials, that arm ATC with (a) anti-CD3 x anti-HER2 BiAb (HER2 BATs) for the treatment of breast and prostate cancer (5, 6), and (b) anti-CD3 x anti-CD20 BiAb (CD20 BATs) for the treatment non-Hodgkin's lymphoma (7) and MM in combination with stem cell transplantation, specific targeting to MM lines by CS1-BATs has not been shown. Armed ATC derived from normal donors not only kill repeatedly, but secrete Th1 cytokines, chemokines (8) and granzyme B when a BiAb bridge synapse is formed between the effector ATC and its target.

METHODS

Approach

The strategy for producing heteroconjugated BiAb for arming ATC involves crosslinking OKT3 with a 10-fold molar excess of Traut's reagent and anti-CS1 (elotuzumab) with a 4-fold molar excess of Sulpho-SMCC according to manufacturer's instructions (9) (step 1), mixing the two cross-linked antibodies overnight at 4°C to produce heteroconjugated CS1Bi (step 2), arming the *ex vivo* expanded ATC with CS1Bi (step 3), and co-culturing the CS1-BATs with MM cell line targets leading to cytotoxicity and cytokine release (step 4).

Activated T Cells

PBMC from normal subjects were obtained with informed and written consent under University of Virginia (UVA) Institutional Review Board (IRB)#18904. PBMC from MM pts were obtained with informed and written consent under UVA Orien IRB HSR 18445 and Wayne State University (WSU) IRB-approved protocol 2008-106 (NCT00938626) (10). PBMC were isolated by Ficoll-Hypaque (Lymphocyte Separation Medium from Corning) and stimulated with OKT3 at 20 ng/ml and expanded in RPMI-1640 containing 10% fetal calf serum and IL-2 (100 IU/ml) as described (8). Unseparated ATCs were armed between 10 and 15 days of culture, most often between 12 and 14 days. Historically, patients' ATC cultures consisted primarily of CD3+ cells, with a small percentage of CD56+ cells. In the phase 1 breast cancer trial, the average composition of 17 patients' ATC products for CD3, CD4, and CD8 cells were 86.7% (+/– 13.5), 52.4% (+/– 15.2), and 34.6% (+/– 15), respectively (5); and for 12 myeloma patients were 94.6% (84.4–98.3), 66.2% (24.8–81.1), and 39.1% (10.2–71.3), respectively (with a mean CD3–/CD56+ of 11.6%, ranging from 0.35 to 63.7) (10).

Multiple Myeloma Cell Lines and Monoclonal Antibodies

The MM cell lines RPMI8226, ARH77, L363, and MM.1S were purchased from ATCC, Manassas, VA. OPM2 was purchased from DSMZ, Germany. OKT3 is an anti-CD3 immunoglobulin G2a (IgG2a) (Miltenyi Biotech). Elo was obtained commercially. OKT3 was chemically heteroconjugated with Elo as described (9).

Quantitative Flow Cytometry-Based Specific Cytotoxicity Assay

First attempts to measure the cytotoxicity of CS1-BATs using standard 4 h ^{51}Cr-release assays showed minimal activity against MM cells even at 25 E:T. Therefore, a more sensitive quantitative assay was developed using flow cytometry in which the concentration of both effector T cells and target cells was measured in fixed volume aliquots (50 μL) before and after 16 h (or more) of culture using an ACEA Biosciences NovoCyte flow cytometer. Target cells are fluorescently labeled with eFfluor 450 (Invitrogen) according to manufacturer's instructions, resuspended at 0.8×10^6 cells per mL, and added to 24 well culture plates in 300 μL of media. T cells are resuspended to provide the designated E:T ratios based on the addition of 300 μL to the target cells. After thoroughly mixing the cells, 120 μL is placed into a counting tube, 7-ADD added, and the cells acquired on the cytometer to establish the baseline E:T ratio. Cells are first gated by forward and side scatter to capture the T cell and myeloma cell line populations. At the final time

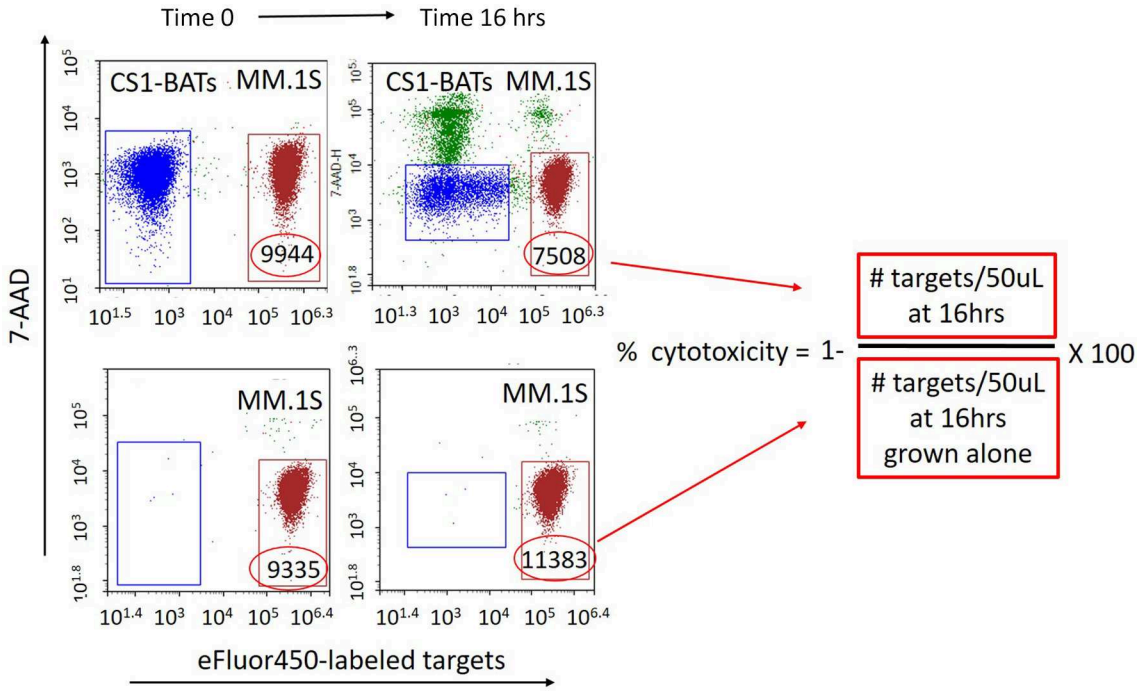


FIGURE 1 | Quantitative flow cytometry-based assay for measuring specific cytotoxicity against multiple myeloma and other non-adherent cell targets. eFlour450-labeled cell targets are cultured alone or in the presence of either BATs or unarmed ATC for 16 h (or longer). The absolute number of target and effector cells in a fixed volume (50 μL) of the test culture is measured upon initial mixing and at a later time point(s) from the same culture. The number of target cells cultured alone is used as a reference for calculating cytotoxicity as a result of co-culturing with ATC. The number of surviving target cells (eFluor450 positive/7-AAD negative) per fixed volume is used to calculate the percent cytotoxicity as [1− (# live targets in ATC co-cultures divided by the number of live targets in parallel cultures grown without ATC)] × 100. Numbers within the gates represent the # of live target cells/50 μL at each time point. *Top left:* BATs plus eFluor450-labeled targets at Time 0. *Bottom left:* eFluor450-labeled targets alone at Time 0. *Top right:* BATs + eFluor450-labeled targets at Time 16 h. *Bottom right:* eFluor450-labeled targets alone at Time 16 h. In this example, cytotoxicity of CS1-BATs against MM.1S myeloma cells is [1-(7508)/11383)] × 100% = 34% at 1:1 E:T.

point, the co-cultured cells are again thoroughly mixed by gentle pipetting prior to sampling. **Figure 1** shows a representative example of the gating used to calculate the specific cytotoxicity directed at MM.1S cell line using 7-AAD (live/dead staining) and eFlour 450-labeled targets. The formula for analysis is as follows: Number of cells/gate are the number of cells per 50 μL of the test culture volume assessed at baseline and at subsequent time points. % cytotoxicity = 1− [# targets incubated with effector T cells at a given time point/# targets at the same time point cultured without effectors] × 100%. Due to the ability to finely measure the E:T ratios in each well, the closest integer value for a donor set is presented in the figures, with the actual range of E:T indicated in the figure legends. For multiday studies, replicate wells are prepared so that the experimental and target-only wells are collected only once at the designated time points.

Quantitation of Cytokines/Chemokines

CS1-BATs or unarmed ATC were co-cultured in the presence of MM cell lines targets, and cytokines, chemokines and granzyme B in the cell-free supernatants were quantitated using the Luminex system. The values are reported in pg/ml (ng/mL for granzyme B) of cell supernatants.

Statistical Analyses

All values are expressed as means ± SD. Mean values were compared using Student's *t*-tests (Prism software) with $p < 0.05$ considered significant for parametric paired samples.

RESULTS

Production of Chemically Heteroconjugated Anti-CD3 × Anti-CS1 (CS1Bi)

The Coomassie stained non-reducing gel in **Figure 2** shows the results of the heteroconjugation. Lane 2 of the gel shows the CS1Bi product adjacent to unconjugated OKT3 (lane 3) and Elo (lane 4). Scanning densitometry of the gel shows 31.2% dimer, 34.2% monomers, and 34.5% multimers.

Antibody and BiAb Binding to MM Cell Lines and ATC

To confirm CS1 expression on MM cells, the MM cell lines were stained with a commercial PE-conjugated anti-CS1 mAb, or Elo followed by secondary staining with PE anti-human IgG. There is clear CS1 expression on the MM.1S myeloma cell line at a concentration of 10 μg/mL of the PE-conjugated

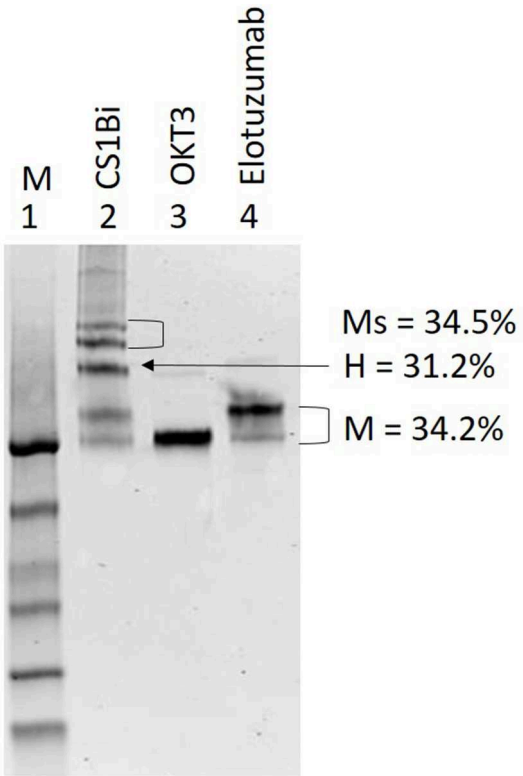


FIGURE 2 | SDS-PAGE of CS1Bi heteroconjugation product. *Lane 1*: molecular weight markers; *Lane 2*: CS1Bi product containing unconjugated monomers, heterodimer, and multimers forms; *Lane 3*: OKT3; *Lane 4:* elotuzumab. M, monomers; H, heterodimers; Ms, multimers.

mAb (**Figure 3A**, left panel); binding of Elo was detected at 16 and 32 μg/mL (**Figure 3A**, right panel). The mean fluorescent intensity (MFI) for anti-CS1 binding for the 5 cell lines is tabulated in **Figure 3A**. Binding of the CS1Bi to MM.1S myeloma cells was detected using a FITC-conjugated anti-murine IgG2a antibody to detect OKT3 in the attached BiAb; OKT3 is barely detectable above the isotype control at 32 μg/ml CS1Bi and is clearly detectable at 64ug/mL (**Figure 3B**). In order to determine the ability of CS1Bi to bind to ATC, ATC were armed with CS1Bi at 500 ng/mL, and also incubated with Elo (2 μg/ml) and anti-HER2 antibody (Herceptin®, human IgG1, 2 μg/ml) as negative controls for human IgG1 binding. Flow cytometry confirms that the CS1 portion of the BiAb can be strongly detected on ATC using PE-anti-human IgG (**Figure 3C**).

Dose Titrations to Determine Optimal Arming Concentration of CS1Bi

In order to establish the optimum arming concentration of CS1Bi, the ATC were left unarmed or armed at 12.5, 25, and 50 ng of CS1Bi/10^6 ATC and tested for cytotoxicity directed at RPMI8226, ARH77, and L363 MM cell lines at a 1 to 1.5:1 E:T (**Figure 4A**). Cytotoxicity directed at RPMI 6226 (**Figure 4A**, left panel) peaked just below 40%. In the ARH77 experiment, the cytotoxicity appeared to plateau around 40% at ≥25 ng of CS1Bi/10^6 ATC arming dose (**Figure 4A**, middle panel). There were no significant differences between the amount of cytotoxicity mediated by ATC armed with 12.5, 25, and 50 ng/10^6 ATC in RPMI6226; however the 50 and 25 ng/million ATC conditions were significantly different than unarmed ATC. The experiments in the L363 cell line showed that the dose titration continued to increase to ∼35% at an arming dose of 50 ng/10^6 CS1Bi and that all arming doses were significantly greater than for unarmed ATC (**Figure 4A**, right panel).

CS1-BATs armed with 50 ng CS1Bi/10^6 ATC were also very effective against MM.1S cells (**Figure 4B,** left panel), while OPM-2 cells were the least sensitive line tested to CS1-BATs at 1:1 E:T, with increased killing at 3:1 E:T (**Figure 4B**, right panel). Although CS1-BATs showed increased cytotoxicity (>90%) at higher E:T, we focused on lower E:T to better distinguish the effects of the arming titration. Thus, the amount of specific cytotoxicity was significantly increased over unarmed ATC in all 5 cells lines ($p < 0.05$). Based on these results, and similar to our other BATs products, the clinical arming dose will be 50 ng of CS1Bi/10^6 ATC.

The ability of elotuzumab and OKT3 alone to redirect the cytotoxicity of ATC was tested by "arming" ATC with each antibody at the same concentration present in the CS1Bi preparation (25 ng per million ATC). **Figure 4C** shows that neither antibody was able to significantly increase the degree of cytotoxicity above that of unarmed ATC against L363 and ARH77 cells.

Cytotoxicity of CS1Bi-Armed PBMC

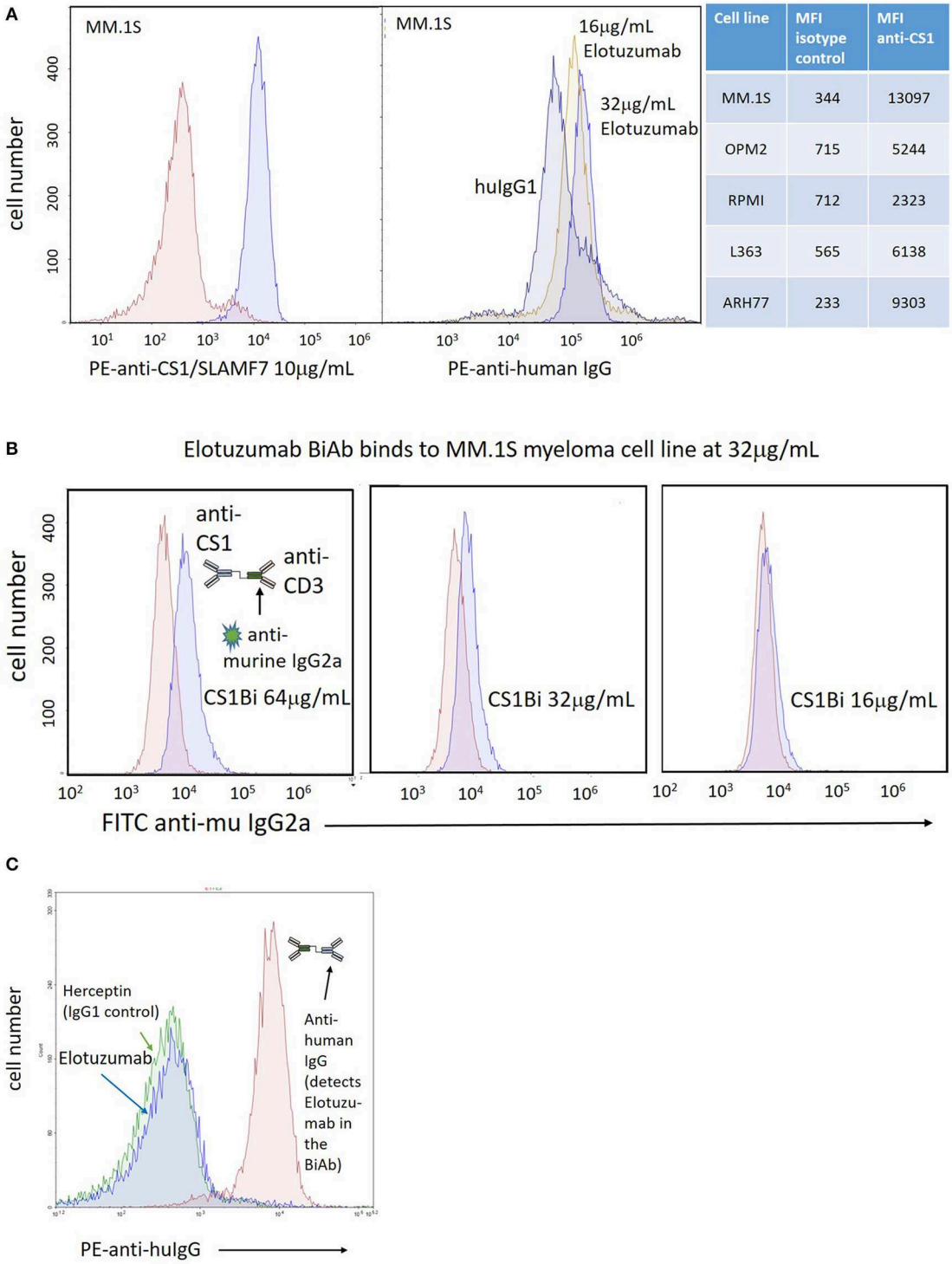
The relative ability of unactivated PBMC to mediate anti-MM cytotoxicity was tested by arming total PBMC at 50 ng/million cells and compared to that of CS1Bi-armed ATC (**Figure 5**). Either BiAb-armed or unarmed cells from 3 donors were added to ARH77 or L363 cells. The percentages of lymphocytes in these 3 PBMC samples were 60, 78, and 82. Against ARH77 cells, the cytotoxicity of the armed vs. unarmed PBMC was unchanged for 2 out of 3 donors, with the third showing an increase in cytotoxicity. This result is similar to what was reported with PBMC armed with anti-Her2 x anti-CD3 BiAb (11). Against L363 cells, 3 out of 3 donors showed significantly higher cytotoxicity when armed with CS1Bi.

Cytotoxicity of CS1 BATs Is Not Blocked by Free Elotuzumab

CS1 BATs were co-cultured with L363 cells in the presence of 100, 600, or 1,200 mcg/mL elotuzumab, which represent concentrations below, at, and above the maximum blood concentration in pts (**Figure 6**). There was no difference between the untreated and elotuzumab-treated groups and all CS1 BATs concentrations were significantly greater than for unarmed cells.

Induction of Th_1 Cytokine and Chemokine Release Upon Binding Target Cells

Overnight co-cultures of unarmed ATC and ATC armed with 50 ng of CS1Bi/10^6 ATC, were performed to assess the induction of Th_1 cytokines, chemokines and granzyme B secretion upon CS1-BATs engagement with RPMI 8226, ARH77, and L363



Cell line	MFI isotype control	MFI anti-CS1
MM.1S	344	13097
OPM2	715	5244
RPMI	712	2323
L363	565	6138
ARH77	233	9303

FIGURE 3 | Expression of CS-1 on MM cell lines. **(A)** *Left panel*: Right peak shows binding of PE-anti-CS1 (Abcam, 95827; clone 162) at 10 μg/mL and 0.4 million MM.1S cells after incubation in 100 μL phosphate buffered saline (PBS)/0.2% bovine serum albumin for 20' at 4°C; Left peak shows binding of PE-isotype control. *Right panel*: Right peaks show relative binding of Elo to MM.1S cells incubated at 16 μg/mL and 32 μg/mL relative to 32 μg/mL human IgG1 (left peak), stained with PE-anti-human IgG (Biolegend, 409304; clone HP6017). The relative binding of the Abcam PE-anti-CS1 vs. isotype control for 5 MM cell lines is shown in the table to the right of the histogram panels. **(B)** Arming titration of CS1-BATs against MM.1S MM cells. Each panel shows binding of CS1Bi by FITC anti-murine IgG2a at either 16, 32, or 64 μg/mL CS1Bi/0.4 million ATC after incubation in 100 μL phosphate buffered saline (PBS)/0.2% bovine serum albumin for 20' at 4°C. The histograms are overlaid against staining of MM.1S by 8, 16, or 32 μg/mL OKT3, respectively, which represents the relative amount of OKT3 in the CS1Bi product. **(C)** Binding of CS1Bi to ATC. CS1Bi was incubated at 500 ng/mL/0.4 million ATC in 100 μL for 20' at 4°C followed by staining with PE-anti-human IgG. Right peak shows the binding to Elo in the bound BiAb. The left peaks show background binding of ATC incubated with Elo or Herceptin (both human IgG1 isotype) at 2 μg/mL/0.4 million ATC.

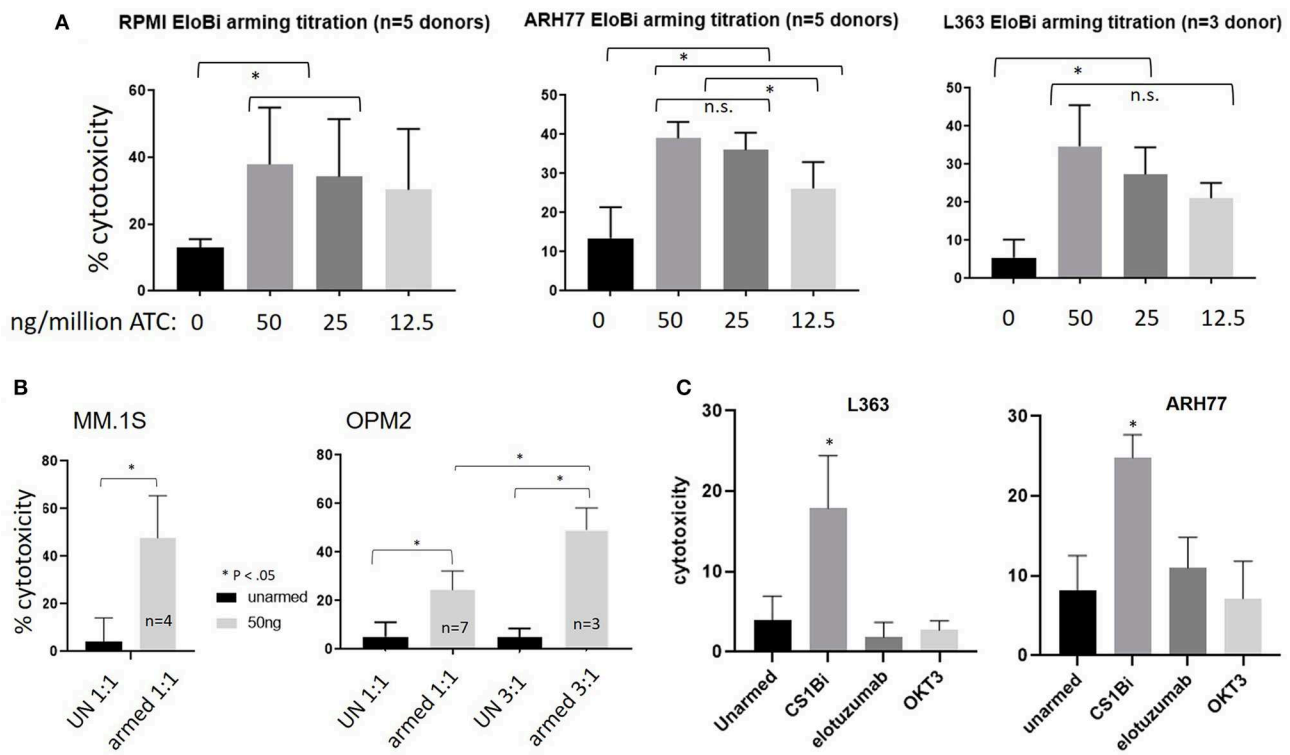


FIGURE 4 | (A) Cytotoxicity of differentially armed BATs. Normal donor ATC were unarmed (UN) or armed with 12.5, 25, or 50 ng/10^6 ATC and tested against RPMI (*Left panel; 1 to 1.5 E:T, n = 5*), ARH77 (*Middle panel; 1.5 to 2.1 E:T, n = 5*) and L363 (*Right panel; 1 to 1.5 E:T, n = 3*) cells in a 16 h cytotoxicity assay. The difference between armed and unarmed ATC (UN) was significant ($p < 0.05$) for 50 and 25 ng/10^6 ATC for all three cell lines, as well as for 12.5 ng/10^6 ATC for ARH77 and L363 cells. *$P < 0.05$. Arming at 50 and 25 ng/10^6 ATC was significantly different than 12.5 ng/10^6 for ARH77 cells. **(B)** Cytotoxicity of CS1-BATs against MM.1S and OPM2 MM cells. Unarmed (UN) BATs or BATs armed with 50 ng/10^6 ATC were incubated for 16 h with MM.1S cells (*Left panel; 0.8 to 1.1 E:T, n = 4)* and with OPM2 cells (*Right panel; 0.7 to 1.36 E:T, n = 7; and 3.1 to 3.6 E:T, n = 3*). MM.1S were highly sensitive to CS1-BATs at 1:1 E:T. OPM2 cells were relatively resistant at 1:1 E:T, with increased cytotoxicity at 3:1 E:T. (*$P < 0.05$). **(C)** ATC were armed with CS1Bi (50 ng/10^6 ATC), elotuzumab (25 ng/10^6 ATC), or OKT3 (25 ng/10^6 ATC) and tested for cytotoxicity against L363 and ARH77 cells at 1:1 to 2:1 E:T in a 16 h flow-based assay. CS1-BATs showed significantly greater killing (*$P < 0.05$) than either unarmed, elotuzumab-armed, or OKT3-armed ATC.

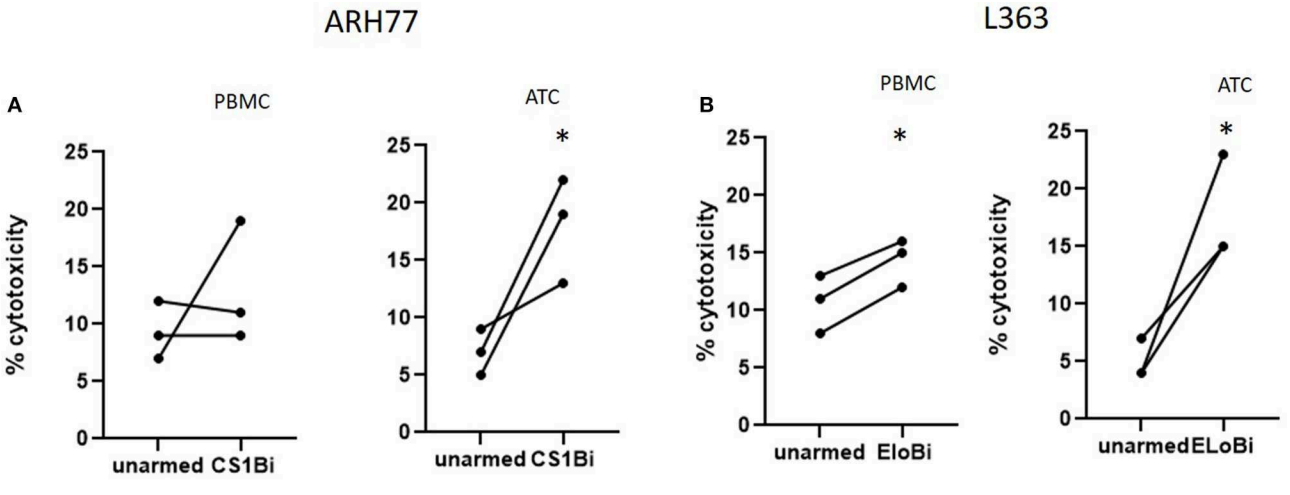


FIGURE 5 | Cytotoxicity of CS1Bi-armed PBMC from normal donors. PBMC and ATC from 3 normal donors were armed with 50 ng/million cells and co-cultured with ARH77 and L363 cells for 16 h at 2:1 E:T. Each donor is represented by a pair of connected dots showing the results of unarmed vs. armed cells. **(A)** CS1Bi-armed PBMC vs. ATC for ARH77 cells. **(B)** CS1Bi-armed PBMC vs. ATC for L363 cells (*$P < 0.05$).

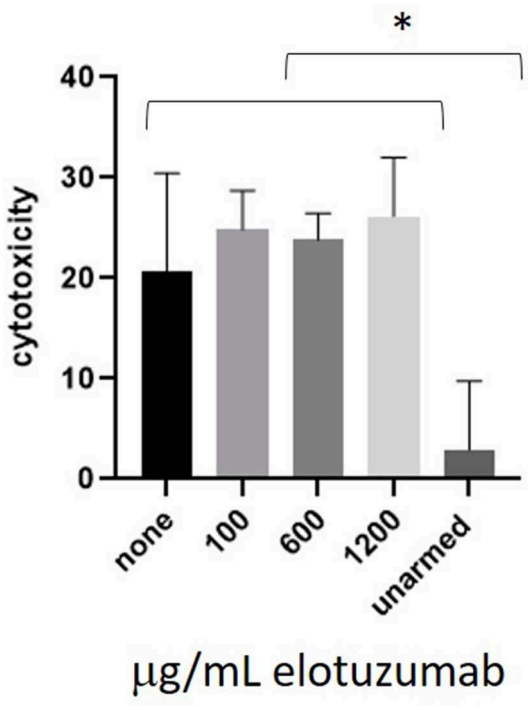


FIGURE 6 | Co-culture of CS1 BATs with L363 cells in the presence of elotuzumab. ATC from 3 normal donors were armed with 50 ng CS1Bi and co-cultured with L363 at an E:T of 1:1 (*$P < 0.05$).

cells. The amounts of IFN-γ, TNF-α, GM-CSF, and granzyme B secreted during a 16 h co-culture increased as a function of CS1Bi arming dose by CS1-BATs produced from 4 normal subjects. (**Figure 7A**). The 50 ng/10^6 ATC arming dose consistently induced more secretion of the respective cytokines and granzyme B vs. unarmed ATC. Significantly elevated levels of chemokines were seen against some of the cell lines for of MIP1-α, MIP1-β, RANTES, and IP10 (**Figure 7B**). CS1-BATs cultured alone did not produce significant amounts of any of the factors tested. *$P < 0.05$.

Specific Cytotoxicity Mediated by ATC of MM Patients

To test whether CS1Bi could trigger ATC produced from cryopreserved PBMC from 4 MM Pts, CS1-BATs armed with 50 ng of CS1Bi/10^6 ATC were tested for cytotoxicity directed at OPM2 and ARH77 MM cell lines at 3:1 and 4:1 E:T, respectively. Significant cytotoxicity was observed against both cell lines, which was comparable to a normal donor (VA05) tested in parallel (**Figure 8**).

Sequential Cytotoxicity by CS1-BATs

In order to show that CS1-BATs are capable of killing multiple times and divide in response to engaging MM cells, CS1-BATs from 3 MM pts were incubated at 1:1 E:T with ARH77 cells supplemented with 100 u IL2/million ATC in the original culture media, and assayed over 3 days to determine the % cytotoxicity and relative number of ATC over time. The % cytotoxicity doubled after 3 days (**Figure 9A**; $p < 0.05$), accompanied by a 2.46-fold increase in the starting concentration of ATC relative to unarmed ATC (**Figure 9B**; $p < 0.05$ for days 2 and 3). The relatively lower cytotoxicity of these samples vs. normal donors at 1:1 E:T (**Figure 4A**) is not unexpected given they were derived from MM patients as well as that 2 out of the 3 PBMC samples had been frozen for 8-9 years. This result shows that upon activation, CS1-BATs are stimulated to divide and are capable of continuous killing. Therefore, even at lower E:T, CS1-BATs can provide an extended effect against MM cells that can be further enhanced through multiple infusions of BATs to promote a cytotoxic anti-tumor microenvironment over time as was seen with HER2-BATs-treated breast cancer pts (5).

DISCUSSION

This study shows that arming ATC with 25–50 ng of CS1Bi/10^6 ATC can generate highly effective cytotoxic T cells directed at CS1 on MM cell lines. *Ex vivo* arming ATC avoids the need to administer large quantities (mg/kg) of BiAb and, more importantly, would likely avoid cytokine release syndrome (CRS) associated with the infusion of anti-CD3 targeting BiAbs. This strategy utilizes humoral antibody targeting to mediate non-MHC restricted cytotoxicity by ATC. Secretion of Th_1 cytokines upon binding of the effector cells to the myeloma cells not only augments tumoricidal activity directed at the malignant B cells, but may increase local cytokine and chemokine secretion that leads to shifting the tumor microenvironment to recruit endogenous immune effectors and induce an endogenous immune response.

Although the chemical heteroconjugation does not produce pure dimers of OKT3 × Elo, the preparation contains enough dimers and multimers to arm ATC converting each ATC into a CS1-targeted CTL. The titration studies determined the "effective" dose of CS1Bi to be 50 ng/10^6 cells. The concentration of anti-CS1 antibody needed to demonstrate the presence of CS1 on the MM cells suggests that CS1Bi is highly effective at triggering BATs against very low amounts of CS1 antigen on the target cells. Flow cytometry data confirmed that the CS1Bi could be easily detected on the ATC. The CS1-BATs were cytotoxic to all 5 MM cell lines even though CS1 is not highly expressed. These observations parallel our earlier study that showed that HER2Bi at an arming dose of 50 to as little 5 ng of HER2Bi/10^6 ATC was not only capable of binding but also mediating specific cytotoxicity and release of Th1 cytokines (IFN-γ, TNFα, and GM-CSF) when the HER2 BATs engaged the HER2 negative cell line MCF-7 (9). An analysis of CS1 expression on MM pts and cell lines showed that most of the cell lines tested expressed less CS1 than pts (12). However, our experience with solid tumor lines does not show a direct correlation between antibody target expression and overall cytotoxicity or release of cytokines, e.g., HER2BATs consistently produce greater amounts of Th1 cytokines against low HER2-expressing MDAMB231 cells than high HER2-expressing SKBR3 cells even though cytotoxicity levels are similar (data not shown). And similar to BATs armed

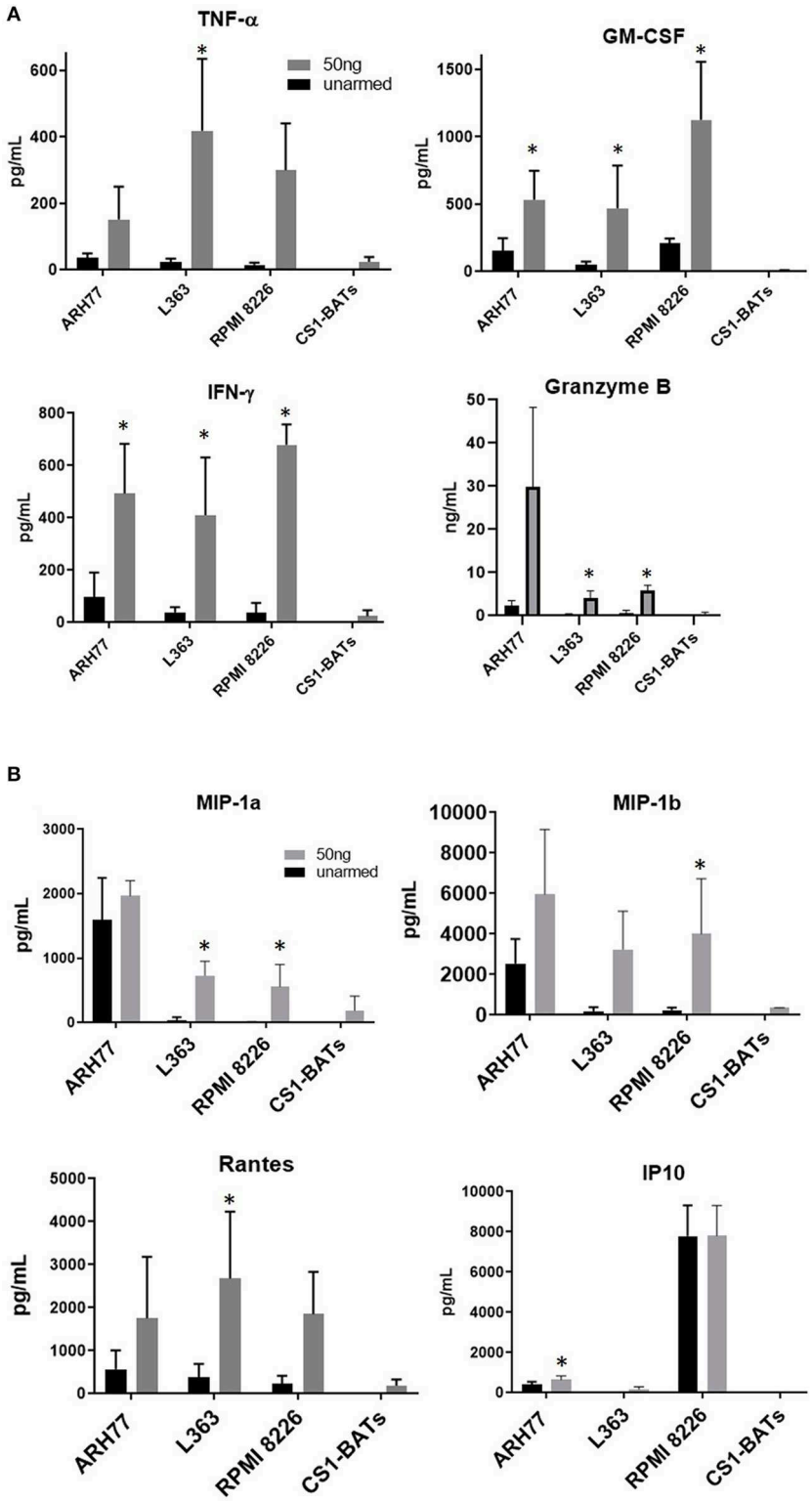


FIGURE 7 | Cytokine production upon engagement of CS1- BATs. Cell-free supernatants from 16 h co-cultures of multiple myeloma cell lines with unarmed ATC, CS1 BATs armed with 50 ng/10^6 ATC at 1:1 E:T (*RPMI, 1.1 to 1.5 E:T; L363, 1.1 to 1.5 E:T)* or 2:1 E:T (*ARH77, 1.1 to 1.8 E:T)* ($n = 4$ donors), or CS1-BATs cultured alone ($n = 3$ donors) were analyzed for production of cytokines, granzyme B, and chemokines. **(A)** Average levels of IFN-γ, TNF-α, GM-CSF (pg/ml), and granzyme B (ng/mL) ($n = 4$ donors) are summarized in the 4 panels. **(B)** Chemokine production by CS1-BATs. The levels of type-1 chemokines (MIP1-a, MIP1-b, Rantes, and IP10) present in the same supernatants as in **(A)** are shown for each of the cell lines tested (pg/mL). *$P < 0.05$.

with anti-CD20 × anti CD3 BiAb (13), CS1 BATs remained active even in the presence of high concentrations of added elotuzumab. The ability of BiAbs to activate armed T cells in response to very low levels of receptor expression and/or differences between the affinity of soluble elotuzumab vs. its affinity as part of the BiAb bound to T cell receptors on the ATC are the likely reasons for maintaining killing in the presence of the targeting monoclonal antibody.

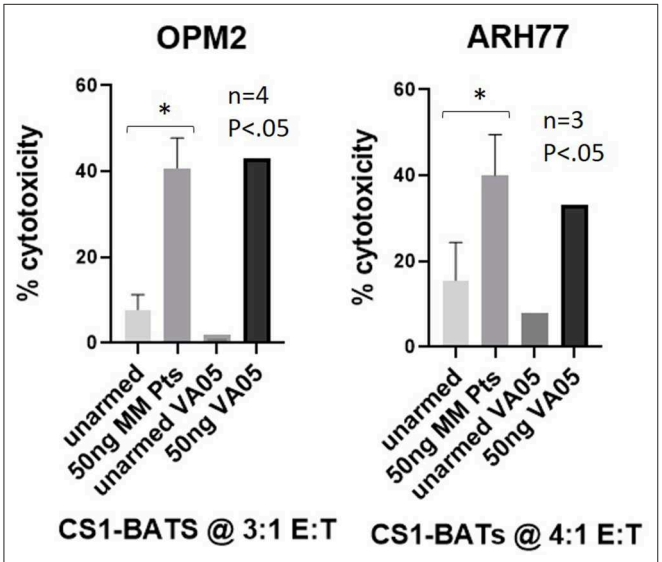


FIGURE 8 | Cytotoxicity of MM pt-derived CS1-BATs. Cryopreserved PBMC from 2 MM pts from WSU and 2 pts from UVA were used to prepare ATC by activation with OKT3 and expansion in IL2-supplemented media for up to 14 days. ATC were armed with CS1Bi at 50 ng/10^6 ATC and co-cultured with OPM2 ($n = 4$) at 2.8–3.6 E:T and ARH77 ($n = 3$) cells at 3.5–4.0 E:T for 16 h. CS1-BATs from a normal donor VA05 (3.2–3.8 E:T) were included as a positive control for each cell line. The level of cytotoxicity of the pt samples was comparable to that of the normal donor performed in parallel (*$P < 0.05$).

Arming of ATC with 25–50 ng of CS1Bi/10^6 ATC was shown to kill MM targets at E:T as low as 1:1. Our prior studies show that ATC exhibit high levels of BiAb-mediated specific cytotoxicity as early as 6 days and as long as 18 days of culture. We have shown that ATC from patients can be armed with anti-CD3 × anti-HER2 BiAb to treat metastatic breast (5) and metastatic prostate (6) cancers, anti-CD3 × anti-CD20 BiAb to treat non-Hodgkin's lymphoma (7, 14) and MM (10), and anti-CD3 × anti-GD2 BiAb to treat neuroblastoma and osteosarcoma (15). All of the BiAb ATC combinations consistently enhance specific cytotoxicity above that seen in unarmed ATC (9, 13, 16, 17). Furthermore we showed that purified CD8 and CD4 populations could be armed with BiAb and mediate specific cytotoxicity, although we did not test T cell populations for antigen-specific cytotoxicity (9). It is clear that BATs are serial killers (8) and persist in the patients for weeks after infusions (5); in the former study, we tested for persistence of the HER2Bi on the surface of the T cells in serial killing assays and showed that the BiAb not only persists on the cell surface, but that a decreasing amount of BiAb on the surface passed on to the dividing daughter cells allows them to use the BiAb to kill again (8). Furthermore, arming with low doses of BiAb enables not only multiple serial killings but also continuous release of IFN-γ, TNF-α, and GM-CSF. The clinical immune evaluation studies in metastatic breast cancer, metastatic prostate cancer, non-Hodgkin's lymphoma and MM showed the induction of Th_1 cytokine patterns with elevated levels of IFN-γ, TNF-α, GM-CSF, and IL-12 in the patients with increases in IP-10, and decreases in IL-8 (5, 6, 10, 18). A more recent study on immune transfer after stem cell transplant showed evidence for not only transfer of established anti-breast cancer immunity but the development of cellular and humoral immunity to other epitopes and well as other tumor antigens (19).

Other approaches to redirect T cell activity to treat MM include bispecific antibodies, non-gene and gene-modified T cell therapies (20–22), and chimeric antigen receptor (CAR)-expressing NK cells (23), from which several clinical trials

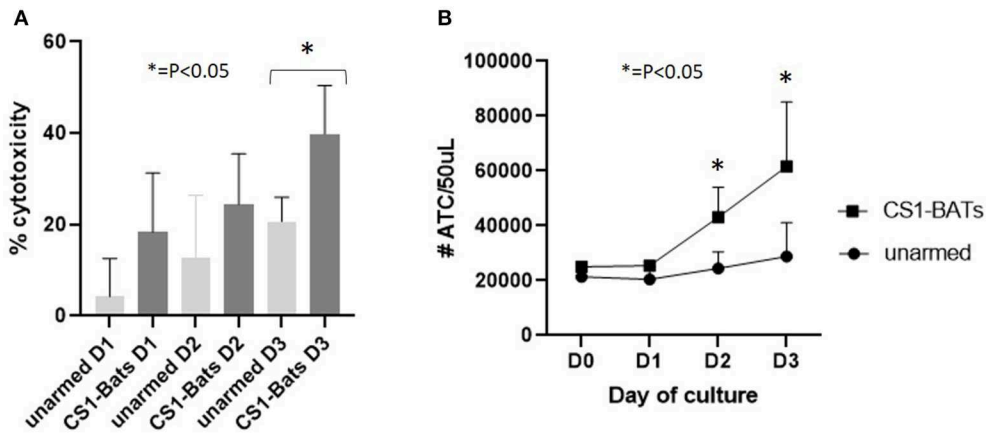


FIGURE 9 | Continuous cell killing and effector cell expansion of MM pt CS1-BATs. CS1-BATs from 3 MM pts were co-cultured with ARH77 target cells at 1:1 E:T (1.1 to 1.5) for 3 days. Duplicate samples for BATs, unarmed ATC, and ARH77 cells grown alone were analyzed for each day of culture to determine relative cytotoxicity and number of ATC for each day of culture. **(A)** Cytotoxicity increased on each successive day, and was statistically different for CS1-BATs vs. unarmed ATC on day 3. **(B)** A similar trend was observed for the total number of ATC present, with the number in CS1-BATs-treated wells significantly higher at days 2 and 3 (*$P < 0.05$).

have shown promising results in terms of response rate and/or duration. As shown with BLINCYTO® and CAR-T products in other hematologic malignancies (24), the major side effects in MM pts have been CRS and neurotoxicity (20, 21) that occur due to the systemic nature of the target cell and difficulties in controlling both the dose and activity of the therapies. Therefore, the CS1-BATs approach is a highly promising alternative for use against MM due to the lack of toxicity demonstrated by previous BATs studies in solid tumors, NHL and MM, combined with the ability to more precisely control potency via adjusting (i) the amount BiAb used to arm the ATC, (ii) the cell dose per infusion, and (iii) the number and frequency of infusions. A clinical trial for refractory MM would be unique in that billions of CS1-BATs could be infused multiple times with minimal toxicities with or without a stem cell transplant in patients with resistant disease with the goal of reducing the tumor burden to attain a MRD status. Such a long-term strategy would lead to not only improved quality life for patients suffering from refractory MM but would lead to potential cures by immunologically eliminating the "last malignant plasma cell" using the endogenous immune system.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was approved by the University of Virginia (UVA) Institutional Review Board (IRB)#18904. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

LL and AT conceived the use of CS1-BATs for treating MM. MH designed, validated, and implemented the flow cytometry-based cytotoxicity assay. AE, LA, and ED contributed to sample preparation and execution of experimental procedures. MH provided methods, data analysis, and figures. LL, MH, and AT authored the manuscript.

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REVIEW
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Bispecific, T-Cell-Recruiting Antibodies in B-Cell Malignancies

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Bispecific antibodies (BsAbs) are designed to recognize and bind to two different antigens or epitopes. In the last few decades, BsAbs have been developed within the context of cancer therapies and in particular for the treatment of hematologic B-cell malignancies. To date, more than one hundred different BsAb formats exist, including bispecific T-cell engagers (BiTEs), and new constructs are constantly emerging. Advances in protein engineering have enabled the creation of BsAbs with specific mechanisms of action and clinical applications. Moreover, a better understanding of resistance and evasion mechanisms, as well as advances in the protein engineering and in immunology, will help generating a greater variety of BsAbs to treat various cancer types. This review focuses on T-cell-engaging BsAbs and more precisely on the various BsAb formats currently being studied in the context of B-cell malignancies, on ongoing clinical trials and on the clinical concerns to be taken into account in the development of new BsAbs.

Keywords: bispecific antibodies, leukemia, lymphoma, myeloma, bispecific T-cell engager, BiTE, clinical development, concerns

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INTRODUCTION

The idea of bispecific antibodies (BsAbs) was initially launched in the early 1960s and the first examples were constructed in 1985 (1). Ten years later, a BsAb (anti-CD19 × anti-CD3) was studied in a clinical trial for the treatment of non-Hodgkin's lymphoma (NHL) (2) and it took until 2009 for the approval of catumaxomab (anti-epCAM × anti-CD3) for the treatment of patients with malignant ascites (3). Advances in protein engineering enable the creation of BsAbs with specific mechanisms of action and clinical applications (4). Although catumaxomab was withdrawn from the market in 2017 for commercial reasons, the excellent clinical results of the bispecific T-cell engager (BiTE), blinatumomab (anti-CD19 × anti-CD3) (5), have renewed the interest and investment in BsAb development.

BISPECIFIC ANTIBODIES

Bispecific antibodies are designed to bind to two different antigens (Ag) or epitopes. These Ags can be present on the same cell, thereby improving the selectivity and binding kinetics of these antibody (Ab) formats. Most BsAbs are developed to bind different targets on different cells, which expand their potential applications. In immunotherapy, they are used to improve tumor cell eradication by bringing cytotoxic cells [T-cells or natural killer (NK)-cells] directly in contact with tumor cells. Given their potential economic value, the pharma industry has taken over their biotechnical

development resulting in more than 100 different formats that have been designed (6). This review tries to focus on different T-cell recruiting formats that have been developed in the treatment of B-cell malignancies.

Effector cell-engaging BsAbs are generally made up of an effector cell-binding domain linked to a tumor Ag-binding fragment. The final format can be made of various known Ab fragments such as single-chain variable fragment (scFv), heavy chain variable domain (VH), light chain variable domain (VL), variable region of a heavy chain of a heavy chain only Ab (VHH), diabody, etc.; or resemble the general architecture of immunoglobulins (Ig). Such fragments provide advantages and disadvantages according to their specific characteristics and properties. Therefore, selection of Ab fragments require careful evaluation, in order to create the most suitable BsAbs for the desired applications (4, 7). One single format is probably not suitable for all applications and BsAbs are generated according to desired characteristics. They differ in terms of size, valency, flexibility, distribution of their pharmacological properties, etc. The two most common forms of BsAbs are the IgG-based and Ab-fragment based formats. IgG-Based BsAb contain an Fc region that helps the stability of the BsAb and the production and purification procedures. Some of the formats of BsAbs currently used for hematological cancers are described in **Tables 1**, **2** and these various formats are shown in **Figure 1**.

Bispecific Antibodies IgG-Like

The Fc domain of an Ig facilitates BsAb purification, improves solubility and stability, extends their *in vivo* half-life (8) and activates several immune cells. When its effector functions are maintained, this Fc region will induce Ab-dependent cell-mediated cytotoxicity (ADCC) by recruiting NK-cells and/or macrophages and complement-dependent cytotoxicity (CDC) by binding the complement (4, 8).

Preferably, CD3-targeting BsAbs require the complete suppression of the Fc-mediated effector functions in order to maximize therapeutic efficacy and to minimize off-target toxicity because binding of Fc to Fc gamma receptor (FcγR) leads to activation of immune effector cells. In reality, the majority of the CD3-targeting BsAbs, currently in clinical practice, have Fc domains with reduced binding activity to FcγR or are BsAb fragments intentionally without the Fc region (9).

However, IgG-like BsAbs composed of two different heavy chains and two different light chains are difficult to produce. The heavy chains of the Bsab can form homodimers (described as heavy chain-pairing problem) and also the light chains can pair to the incorrect heavy chains (light chain-pairing problem). Different solutions have been proposed to avoid these undesired mispairs and some of them are integrated in **Table 1**. A major progress in this field was the development of the "knobs-into-holes" (KiH) strategy that consisted of introducing large amino acid side chains into the CH3 domain of one heavy chain that fit into an appropriately designed cavity in the CH3 domain of the other heavy chain (10).

Bispecific Antibodies Without Fc Region

BsAbs lacking an Fc region can be produced by linking two different single-chain antibodies with a linker. Their Ag-binding part contain only the variable regions of the heavy and light chains connected to each other by a linker (**Table 2**). They are smaller than the bispecific molecules with an Fc region, and this reduced size results with increased tissue penetration, but also fast renal elimination resulting in a short plasma half-life. This reduced circulation time requires more frequent administrations or continued infusion (11, 12). The half-life can be extended using different engineering technologies, such as coupling to inert polymers (polyethylene glycol) (13) adding an Fc part (14), attaching an albumin-binding part (15) or even immunoglobulin-binding domains (16). Companies are currently introducing these half-life extended BsAb in order to limit the administration frequency and improve patients' comfort. Prospective clinical studies will investigate the efficacy and toxicity of these conjugated BsAbs and allow a comparison with the original BsAbs (e.g., AM701, an anti CD3 × BCMA BiTE) is a half-life extended version of AMG420 that showed promising results in the first phase I trial).

RECRUITMENT OF EFFECTOR CELLS

Main Ag for Targeting T-Cells: CD3

BsAb constructs guide immune effector cells to tumor cells by cell-specific receptors such as CD3 on T-cells or CD16 on NK-cells. Currently, approximately half of the evaluated BsAbs by clinical trials are BsAbs that recruit T-cells (17). Their mechanism of action is based on the activation of T-cells by binding CD3ε in the T-cell receptor (TCR) complex irrespective of major histocompatibility complex (MHC) restriction or TCR epitope specificity. Although required for their anti-cancer activity, the binding to the antigen may lead to an excessive immune reaction with activation of bystander immune cells and non-immune cells that finally results in a cytokine release syndrome (CRS).

Most T-cell engaging BsAbs aim to bind CD3ε to guide T-cells to the target cells. An alternative Ag, CD5, has been previously explored (18) but the observed responses were inferior to those obtained with CD3ε-binding BsAbs. Unfortunately, CD3 will recruit different types of T-lymphocytes (including immune-suppressive ones) that can limit their efficacy. For example, Duell et al. (19) showed that blinatumomab also activates regulatory T-cells (Tregs), who inhibit cytotoxic T-cell proliferation, thereby preventing tumor cell destruction. As a result, usage of NK-cells instead of T-cells draw attention in clinical development (see **Table 3**) (17).

CD3-based BsAbs targeting T-cells also demonstrated other disadvantages, such as (1) potentially high toxicity, particularly for targets with wide tissue expression; (2) partial tumor destruction and the development of resistance to treatment due Ag escape (8) and rapid and powerful activation of a large pool of T lymphocytes that is no longer counterbalanced by TCR regulation (20, 21). The interest in this type of BsAbs renewed after the first clinical results obtained with blinatumomab (see section "BiTE anti-CD19 – CD3") (22). Impressive responses

TABLE 1 | Ab formats used for hematological cancers: Bispecific antibodies IgG-like.

Name/Platform	Firm	Characteristics	Heavy chain engineering		Light chain engineering	Fc domain	Production	Remarks	References
			"Knob-in-hole" technology	Other strategies					
BsAb armed activated T-cells (BAT)	Mostely academic	Combination of an mAb targeting the tumor Ag with an mAb targeting the effector cells	No	No	No	Functional Fc	Chemical heteroconjugation of 2 mAbs	Combined with *ex vivo* activated T-cells	161
CrossMab	Roche	Exchange of either the constant domain, variable domains or the whole Fab fragment	Yes	Electrostatic steering	Crossover of an existing fragment without the need for the identification of common light chains	Fc part without effector function	Almost natural, full-sized humanized IgG1 antibody	Not immunogenic, also applied to 2 + 1 and 2 + 2 formats	162, 163
Veloci-Bi	Regeneron	Common light chain approach combined with mutation of protein A binding site for improved purification	No	Selection of correct heterodimers by Protein A affinity chromatography using a new protein A resin	Use of heavy chains that employ identical light chain	Fc part without effector function	Recombinant production, purification enables identification of correct heterodimers	Not immunogenic	164
SEEDbodies		Specific pairing through the design of alternating segments from human IgA and IgG	No	Strand-exchange engineered domain: interdigitating β-strand segments of human IgG and IgA C_H3 domains	Additional engineering for correct heavy-to-light chain pairing	Fc part without effector function	Recombinant production	SEEDbodies assure correct Heavy chain pairing, but additional engineering of light chains can be necessary	165
Biclonics	Merus	Charge pairs in the CH3 that favor heterodimerization	No	Introduction of charged residues at different positions within the Fc part	Fab fragment consisting of common light chain fragments	Fc part without effector function	VH genes cloned in the backbone IgG1; Recombinant production of full IgG	/	166, 167
XmAb	Xencor	Typically, scFv fused to one Fc instead of Fab fragment to enable bispecificity	Yes	Set of minor and precise changes to the Fc region leading enhanced heterodimerization Improved purification procedure	Different formats exist: Fab or ScFV	Fc part without effector function	Recombinant production and purification by l protein A affinity chromatography	Full-sized humanized IgG1 Ab, nearly identical to natural Ab (similar structure and sequence)	168
Duobody	Genmab	Controlled Fab-arm exchange (cFAE) from two parent homodimeric antibodies	Yes	Fc silent mutations	Separate expression and purification of the 2 component antibodies followed by assembly into BsIgG	Fc activity can be retained or silenced depending on the characteristics desired	Almost natural, full-sized humanized IgG1 antibody	Full-sized humanized IgG1 Ab, minimal modifications to the native Ab structure	169
TriFAb (Trifunctional Ab)	TRION	Produced from two half antibodies from parental mouse IgG2a and rat IgG2b isotypes	No	/	Species−restricted heavy/light chain pairing	Fc part with effector function	Produced using the quadroma technology and captured by protein A affinity chromatography	Trifunctional ≥ Highly immunogenic and toxic (CRS)	170

TABLE 2 | Ab Formats used for hematological cancers: Bispecific antibodies with single chain formats.

	Characteristics	Molecular Weigth	Half life	Linker	Administration	Remarks	References
BiTE	2 scFv fragments, connected by flexible linker peptides	~55 kDa	2 h	15–amino acid (G4S1)3 (single-letter amino acid code) linker	Continuous infusion	Rely exclusively on effector-tumor synapse formation	171
BiKE	BiKEs: 2 scFv fragments, connected by flexible linker peptides are similar in design to BiTEs but they target CD16 on NK-cells	58–60 kDa	ND	20-amino acid segment of human muscle aldolase	ND	Not immunogenic, further expansion of NK-cells (TriKE)	172, 173
TriKE	TriKEs consist of a BiKE into which IL-15 was subsequently sandwiched	~96 kDa	ND	Human IL-15 with N72D substitution, flanked by two flanking sequence	ND	Mutated form of IL-15 expands NK-cells	173
Diabodies	A single−chain format based on 2 peptides, each one contains a heavy chain variable region (VH) for an Ag recognition site paired with a light chain variable region (VL) of a second Ag recognition site	58 KDa	2 h	15 amino acids with sequence GGGGSGGRASGGGGS	Frequent injections or infusions	Variants of diabodies consist of dual-affinity retargeting molecules (DART) or tetravalent constructs that combine two diabodies (TandAb)	174

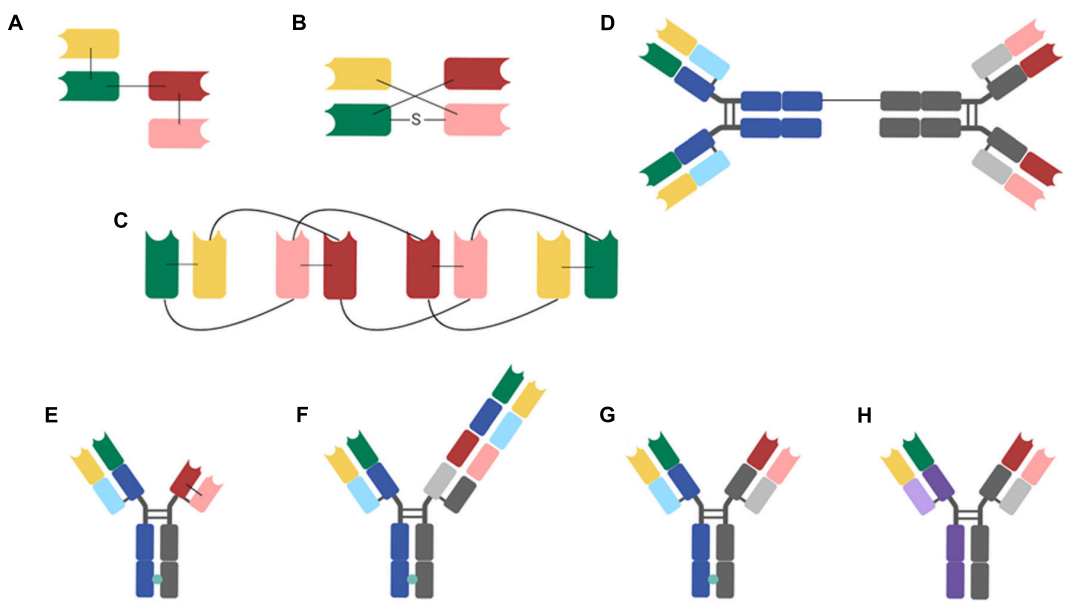

FIGURE 1 | BsAb formats studied for hematological B-cell malignancies **(A)**, BiTE (Tandem scFvs); **(B)** DART; **(C)** TandAb (Tandem diabodies); **(D)** BAT; **(E)** TDB: Xmab (scFv-Fab IgG); **(F)** TCB: CrossMAb; **(G)** TDB: DuoBody; **(H)** TriFAb (Rat-mouse hybrid IgG). The different antibody domains are as follows: green, variable region of heavy chain 1 (VH 1); red, variable region of heavy chain 2 (VH 2); yellow, variable region of light chain 1 (VL 1); pink, variable region of light chain 2 (VL 2); light purple, constant region of light rat chain; dark purple, heavy chain of immunoglobulin G2b (IgG2b); light blue and light gray, constant regions of light mouse chain; dark blue and dark gray, heavy chains of mouse IgG2b; turquoise circles, Knob-in-Hole (KiH) BiTE, bispecific T-cell engager; DART, dual-affinity re-targeting; Fab, Fab region; S, disulfure; scFv, single-chain variable fragment; TandAb, tandem diabody; TDB, T-cell-dependent bispecific antibody; TriFAb, trifunctional antibody, triomab.

TABLE 3 | Clinical development of BsAbs (selected trials).

Names (Sponsors)	Targets (diseases indications)	Format	Phase (NCT#)	References
T-cell redirection				
AMG420, BI 836909 (Boehringer Ingelheim, Amgen)	CD3 × BCMA (MM)	Tandem scFv (BiTE)	Phase I (NCT02514239, NCT03836053)	102, 175
AMG701 (Amgen)	CD3 × BCMA (MM)	Tandem scFv-scFc(G1) (HLE-BiTE) Possibly Fc-silencing	Phase I (NCT03287908)	176
CC-93269, EM901 (Celgene)	CD3 × BCMA (MM)	Fab-Fc(G1) × Fab-Fab-Fc(G1) (CrossMab in the 2 + 1 format) Possibly Fc-silencing	Phase I (NCT03486067)	103
JNJ-64007957 (Janssen)	CD3 × BCMA (MM)	Hetero H, HL exchanged IgG4 (DuoBody) Possibly Fc-silencing	Phase I (NCT03145181)	177
PF-06863135 (Pfizer)	CD3 × BCMA (MM)	Hetero H, HL assembly IgG (DuoBody) Possibly Fc-silencing	Phase I (NCT03269136)	106
REGN5458 (Regeneron)	CD3 × BCMA (MM)	Hetero H, cL IgG4 Possibly Fc-silencing	Phase I/II (NCT03761108)	178
AMG424, Xmab13551 (Amgen)	CD3 × CD38 (MM)	Fab-Fc(G1) × scFv-Fc(G1) (Xmab) Possibly Fc-silencing	Phase I (NCT03445663)	97
GBR 1342 (Glenmark)	CD3 × CD38 (MM)	Fab-Fc(G1) × scFv-Fc(G1) (Xmab) Possibly Fc-silencing	Phase I (NCT03309111)	98, 179
RG6160, RO7187797, BFCR4350A (Genentech)	CD3 × FcRH5 (CD307) (MM)	Hetero H, HL assembly IgG1, IgG assembled from half-antibodies	Phase I (NCT03275103)	35
JNJ-64407564 (Janssen)	CD3 × GPRC5D (MM)	Hetero H, HL exchange IgG4 (DuoBody) Possibly Fc-silencing	Phase I (NCT03399799)	109
Vibecotamab, Xmab14045 (Xencor)	CD3 × CD123 (B-ALL, AML, CML)	Fab-Fc(G1) × scFv-Fc(G1) (Xmab) Possibly Fc-silencing	Phase I (NCT02730312)	180, 181
A-319 (Generon)	CD3 × CD19 (B-cell lymphoma)	scFv-Fab (ITab)	Phase I (NCT04056975)	182
MGD011, JNJ-64052781 (Janssen)	CD3 × CD19 (NHL, B-ALL, CLL)	DART	Phase I: Withdrawn (NCT02743546)	85
AFM11 (Affimed)	CD3 × CD19 (ALL, NHL)	Tandem diabodies (TandAb)	Phase I: Suspended (NCT02106091 and NCT02848911)	86
AMG562 (Amgen)	CD3 × CD19 (NHL)	Tandem scFv-scFc(G1) (HLE-BiTE) Possibly Fc-silencing	Phase I (NCT03571828)	183
Blinatumomab, Blincyto, MT103, MEDI-538, AMG103 (Amgen)	CD3 × CD19 (B-ALL, NHL, MM)	Tandem scFv (BiTE)	Marketed (ALL), Phase I/II [NCT01741792 et NCT02811679 (NHL), NCT03173430 (MM)]	5, 83, 184, 185
GEN3013 (Genmab)	CD3 × CD20 (NHL)	Hetero H, HL exchanged IgG1 (DuoBody) Possibly Fc-silencing	Phase I/II (NCT03625037)	186
Mosunetuzumab, RG7828, RO7030816, BTCT4465A (Genentech)	CD3 × CD20 (CLL, NHL)	Hetero H, HL assembly IgG1, IgG assembled from half-antibodies Possibly Fc-silencing	Phase I/II (NCT03677141 and NCT03677154)	187, 188
Plamotamab, XmAb13676 (Xencor)	CD3 × CD20 (CLL, NHL)	Fab-Fc(G1) × scFv-Fc(G1) (Xmab) Possibly Fc-silencing	Phase I (NCT02924402)	189
REGN1979 (Regeneron)	CD3 × CD20 (ALL, CLL, and NHL)	Hetero H, cL IgG4 Possibly Fc-silencing	Phase I/II (NCT03888105, NCT02290951)	90, 91
RO7082859, RG6026, CD20-TCB (Hoffmann-La Roche)	CD3 × CD20 (NHL)	Fab-Fc(G1) × Fab-Fab-Fc(G1) (CrossMab in the 2 + 1 format) Possibly Fc-silencing	Phase I (NCT03075696)	93
FBTA05, Lymphomun (Technical University of Munich)	CD3 × CD20 (CLL, NHL)	Trifunctional Ab (TriFAb)	Phase I/II (NCT01138579): Terminated	87, 89, 170
CD20Bi (Barbara Ann Karmanos Cancer Institute)	CD3 × CD20 (NHL)	BAT	Phase I (NCT00244946)	190, 191

(Continued)

TABLE 3 | Continued

Names (Sponsors)	Targets (diseases indications)	Format	Phase (NCT#)	References
NK-cell redirection				
AFM13 (Affimed)	CD16A × CD30 (NHL, HL)	Tandem diabodies (TandAb)	Phase I/II (NCT02321592, NCT03192202 and NCT04101331	24, 192
Immune cell redirection				
INBRX-105 (Inhibrx)	PD-L1 × 4-1BB (NHL, HL)	Tandem VHH-Fc(G1) Possibly Fc-silencing	Phase I (NCT03809624)	193
Targeting tumor heterogeneity				
OXS-1550, DT2219ARL (Masonic Cancer Center, University of Minnesota)	CD19 × CD22 (B-cell lymphoma and leukemia)	Tandem scFv fusion protein (BiTE fused to modified diphtheria toxin)	Phase I/II (NCT02370160, NCT00889408)	132, 194
Targeting multiple checkpoints				
MGD013 (Macrogenics)	PD-1 × LAG3 (Solid and Hematological malignancies)	Tandem domain-exchanged Fv-Fc(G4) (DART-Fc)	Phase I (NCT03219268)	142
KN046 (Alphamab)	PD-L1 × CTLA4 (Solid and hematological malignancies)	Hetero H, cL IgG1	Phase I (NCT03733951)	195
Targeting checkpoint and tumor antigen				
TG-1801, NI-1701 (TG Therapeutics)	CD47 × CD19 (B-cell lymphoma)	cH IgG1 (κλ body)	Phase I (NCT03804996)	196

Data available as of November 05, 2019. Molecules are classified based on target Ags. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoblastic leukemia; BAT, Bispecific antibody armed activated T-cells; BCMA, B-cell maturation antigen; BiTE, bispecific T-cell engager; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DART, dual-affinity re-targeting; Fab, antigen-binding fragment; FcRH5, Fc receptor homolog 5 (CD307); GPRC5D, G protein-coupled receptor family C group 5 member D; H, heavy; HL, Hodgkin lymphoma; HLE, half-life extended; Ig, immunoglobulin; ITab, immunotherapy antibody; L, light; LAG3, lymphocyte-activation gene 3; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NK, natural killer; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand; scFc, single-chain Fc fragment; scFv, single-chain variable fragment; TandAb, tandem diabody; TriFAb, trifunctional antibody, triomab; VHH, heavy chain-only variable domain.

were observed at very low doses in patients with NHL who received blinatumomab via a continuous intravenous infusion to reach the desired minimum concentrations (22). In addition, an exceptional complete response rate of 43% was reported in the first studies on relapsed/refractory (r/r) acute lymphoblastic leukemia (ALL) (23).

Main Ag for Targeting NK-Cells: CD16A

An alternative to T-cell usage consists in activating and directing NK-cells to malignant cells. Compared to T-cells, NK-cells are not subjected to HLA restriction. In addition, NK-cell therapies may be better tolerated by patients than their T-cell counterparts (24). Several receptors capable of activating the cytotoxic function of NK-cells have already been described, notably CD16, NKp30, NKp46, NKG2D and DNAX Accessory Molecule-1 (DNAM-1) (25, 26). Contrary to other activating receptors present in human NK-cells, CD16 can strongly trigger activation without co-stimulatory receptors. There are two isoforms of CD16 in humans, CD16A and CD16B, having a low affinity receptor for IgG Fc domain. CD16A is expressed in NK-cells, macrophages and placental trophoblasts, whereas CD16B is expressed in neutrophils. Only the CD16A isoform is capable of triggering both IL-2 secretion and tumor cell destruction (27).

Despite its advantages, CD16 is often cleaved on the surface of NK-cells by a disintegrin and metalloptroteinase-17 (ADAM17) which likely results in a decrease in the activities mediated by this receptor (28). To address this concern, combining a BsAb and ADAM17 inhibitor was evaluated and showed improved therapeutic efficacy (29). An alternative solution is targeting other receptors on the NK–cells, alone or in parallel to CD16. Recently, the group of E Vivier showed the increased cytotoxic effect of targeting two activating receptors, NKp46 and CD16, on NK-cells (30).

Lastly, in addition to directing the cytotoxicity of the NK-cells, improvements were made to their survival and proliferation. IL-15 was incorporated into a Bispecific Killer cell Engager (BiKE) structure to create a Trispecific Killer cell Engager (TriKE) which was confirmed to have the capability to enhance NK-cell cytotoxicity with improved survival and proliferation *in vitro* (31).

BINDING TO TUMOR CELLS

Various parameters will influence the effectiveness of the BsAbs activity. The major factors that determine whether an Ag is a good target include (1) tumor-specificity and absence on healthy tissues (32), (2) prevalence and level of expression on tumor cells (32), (3) potential expression on malignant precursor or stem cells (33), and (4) low levels of circulating, soluble forms.

Moreover, the cytotoxic potential of BsAbs is affected by the target Ag size and the distance between the epitope and the target cell membrane (34, 35). For example, if the distance between the epitopes is large, inhibitory molecules can interfere with the formation of the synapse (35). To achieve optimal effector cell activation, the affinity of the monoclonal Ab, the location of the target epitope in the antigen (Ag) and the Ag density on the surface of the target cells must be taken into account (10).

Furthermore, the low number of truly tumor-specific cell surface molecules limits the use of BsAbs in to cancers where the target Ag is highly overexpressed in malignant cells compared to healthy cells and when the related toxicity toward healthy cells is clinically tolerable (36). Most BsAbs in clinical development target well-known B-cell Ags, particularly the CD19, CD20, CD38, CD123, or B-Cell Maturation Ag (BCMA). These targets are generally also expressed by normal plasma cells and B-cells. Nevertheless, depletion of these cells can be tolerated without inducing serious clinical side effects (17). In addition, these targets are specific for hematopoietic lineage and are not expressed in other normal tissues, which helps to reduce off-tumor activity and side effects.

B-CELL MALIGNANCIES

The B-cell subtypes and the various associated malignancies as well as the different Ags expressed in the B-cell lineage are shown in **Figures 2**, **3**.

Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a hematological malignancy induced by proliferation and accumulation of immature lymphoblasts in various tissues. It is seen in both pediatric and adult patients, showing a bimodal distribution (37). While young patients have a good prognosis, the outcome for adults can be dismal (38, 39). Its prognosis depends further of other factors, such as age, chromosomal abnormalities, genetic alterations and the implicated cell lineage. Although, ALL can be derived from NK-cell, T-cell and B-cell lineages, the majority of the disease is associated with B-cell precursors (40, 41). Chromosomal abnormalities play a critical role in development of ALL. The Philadelphia chromosome (Ph) or translocation *t*(9;22), is a critical anomaly that determine the characteristic of the disease, yielding poor prognosis (42, 43). Initially, patients are diagnosed based on the abundance of lymphoblasts (>20%) in bone marrow or blood (44). Since ALL is associated with premature B-cells, B-cell specific differentiation markers; CD19, CD20, and CD22, are highly associated Ags that are used for diagnosing and targeting with immunotherapeutic agents (40, 45).

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a hematologic disorder defined by accumulation of monomorphic mature B-cells within blood, bone marrow, lymph nodes, and spleen (46). It is observed with a median age at diagnosis of 70 and male:female ratio of 1.5 (47).

Chronic lymphocytic leukemia progression is driven by various genetic abnormalities. Somatic mutations, such as TP53, BIRC3, NOTCH1, ATM, and SF3B1 disrupt pathways including DNA damage, cell cycle control, NOTCH signaling and mRNA processing (48–50). Deletion of chromosome 13 (loss of miR-15a and miR-16-1) and trisomy 12 are the most common chromosomal aberrations observed in CLL, triggering tumorigenesis. Secondary abnormalities are observed at the later stages of the disease, causing resistance to therapy. Essentially, the presence of mutations or deletions in the p53 gene and the mutation status of the immunoglobulin heavy-chain variable region gene (IGH) are strong indicators of poor prognosis (51–54).

Chronic lymphocytic leukemia is well characterized by the expression of CD5 and CD23 along with B-cell markers CD19, CD20, together with high abundance of a single light chain (κ or λ), due to clonal B-cell amplification (46). The diagnosis is obtained by immunophenotyping and blood count of B-cells. If monoclonal B-cells are more than 5000 cells per μL, the diagnosis of CLL is retained (55).

Multiple Myeloma

Multiple myeloma (MM) is an incurable malignancy, caused by monoclonal proliferation of non-functional plasma cells in the bone marrow (56). The median age at diagnosis is 69 years with median overall survival of 8.5 years for transplant-eligible patients (57). Although good response rates are observed with initial therapy, the disease relapses and no longer responds to therapy, causing poor prognosis (56).

Multiple myeloma is characterized by the secretion of monoclonal immunoglobulins or light chains (described as M-protein). Initially, it is a benign disorder where 5 to 10% can evolve into a symptomatic malignancy (58, 59). This progression is driven by a clonal evolution within malignant plasma cells. The genomic infrastructure of MM is highly heterogeneous. Although, the events leading to MM transformation are unclear, numerous genetic abnormalities contribute to disease progression. Disruption of regulation of cyclin D and IgH proteins, including translocations *t*(11;14) and *t*(4;14), are common chromosomal abnormalities observed in early stages, together with hyperdiploidy located in odd chromosomes (60, 61). During progression, as the disease advances, the genetic stability decreases. Additional aberrations, such as chromosomal loss/gains, somatic mutations (KRAS, NRAS, and TP53), hypermethylation and more translocations (MYC), trigger further oncogenic events (62, 63).

An initial diagnosis is assessed by monoclonal protein level, bone marrow biopsy, radiologic imaging and is based on the presence of symtoms (annotated by the acronym CRAB: hypercalcemia, renal failure, anemia and bone lesion) (64, 65). Furthermore, the disease stage can be obtained by International Staging System (ISS) (66), revised on 2015 with additional genetic risk factors (67). Although there is no specific gene marker for MM, Ags such as CD38, BCMA, and CS1/SLAMF7, are currently targeted by immunotherapeutic strategies (68).

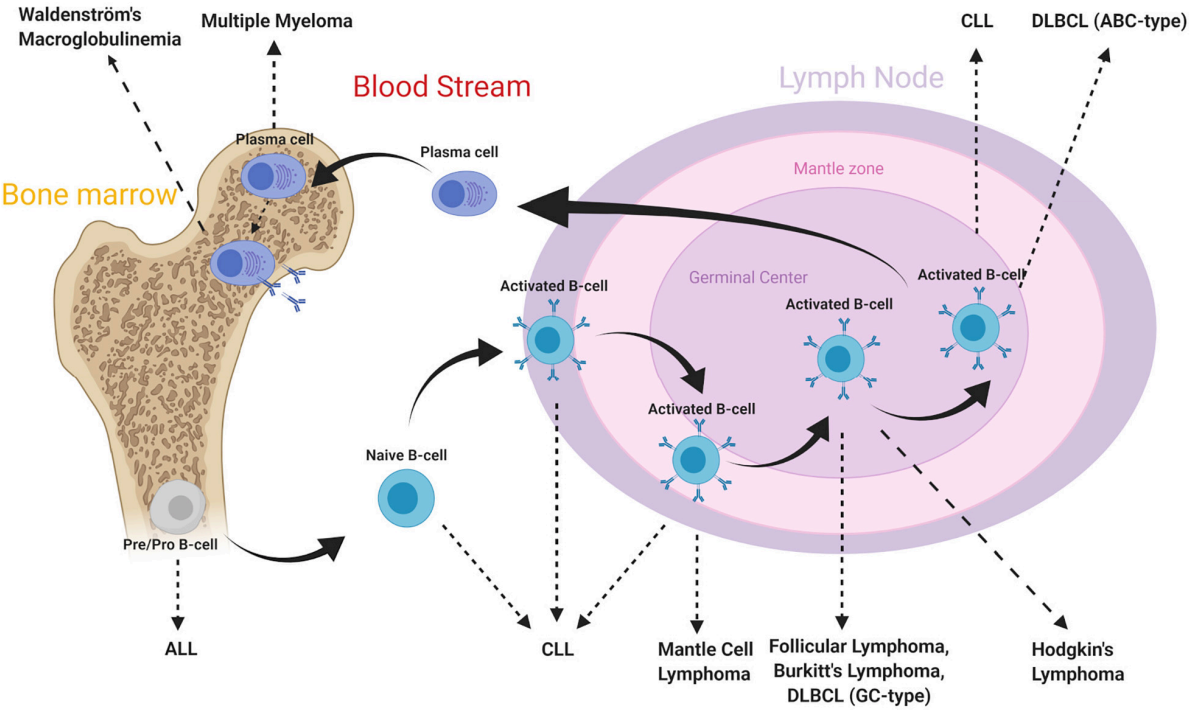

FIGURE 2 | B-cell subtypes and associated malignancies. From Hematopoietic stem cell differentiation into myeloid and lymphoid lineages. After Ag-binding, B-lymphocytes further mature in lymphoid tissues where they undergo various morphological, genetic, and chromosomal alterations. As a consequence, various cell surface Ags reside on cell membrane along maturation process. Disruptions in these mechanisms may lead to the development of malignancies. The B-cell malignancies are divided into subgroups based on location, subtype and activation state of B-cells. This figure is adapted from (70, 159).

Non-Hodgkin Lymphomas

Non-Hodgkin lymphomas are B-cell malignancies that are primarily located in lymph nodes. The disease progression is driven by precursor lymphocytes, where 85% of the cases emerge from B-cell precursors (69). The 5-year survival rates vary highly, from 30% to 86%, among the subtypes of NHL (70). These subtypes are mainly categorized into two groups. Aggressive lymphomas are rapidly evolving entities with a high tumor cell proliferation rates, but potentially curable when responding to high-dose chemotherapy. In contrast, indolent subtypes represent low grade lymphomas and are incurable (71).

Specific translocations enhance the expression of oncogenic proteins and disrupt DNA damage control mechanisms and will finally result in the development of various NHL subtypes (69). To target these cells, cell surface Ags CD19, CD20 and CD30 are widely used targets (72).The diagnosis is established by tissue biopsy, followed by immunohistochemistry and genetic studies (71). Further evaluation of the disease progression can be obtained by staging systems, such as international prognostic index (IPI) and combined Positron Emission Tomography – Computed Tomography (PET-CT) (73).

Treatment Strategies for B-Cell Malignancies

For fit patients, the combination of chemotherapy with corticosteroids remains the first line treatment for most of the listed malignancies. The anti-CD20 monoclonal Ab rituximab will be added for patients with CLL, B-cell NHL, and ALL. Patients that are ineligible for chemotherapy will be treated with specific pathway-inhibitors, such as Bruton tyrosine kinase (ibrutinib), B-cell lymphoma 2 (bcl-2) inhibitors (venetoclax), proteasome inhibitors (bortezomib, carfilzomib) or immunomodulating agents (lenalidomide, pomalidomide). For MM and Hodgkin lymphoma, monoclonal Abs are currently approved in the relapsed setting: daratumumab is the monoclonal Ab that binds to CD38, while brentuximab-vedotin is an Ab-drug conjugate that recognizes CD30. Autologous stem cell transplantation (SCT) will be performed at diagnosis for patients with MM or at relapse for NHL patients.

The efficacy of the initial therapy is evaluated by specific disease parameters and by minimal residual disease (MRD) status. MRD evaluation being negative is a strong indicator of prognosis-free survival while being positive suggests potential relapse (74). In case of disease relapse, a second line therapy is applied. Depending on the cancer type and relapse time, salvage therapy includes more and more specific pathway inhibitors that will be used in combination or in monotherapy. MRD determination has clinical implications in the treatment for ALL, where only MRD positive patients will undergo allogeneic SCT.

Current developments in immunotherapy, such as T-cell engaging BsAbs and chimeric Ag receptor T-cells (CAR-T), show promising results in the first clinical studies to enhance

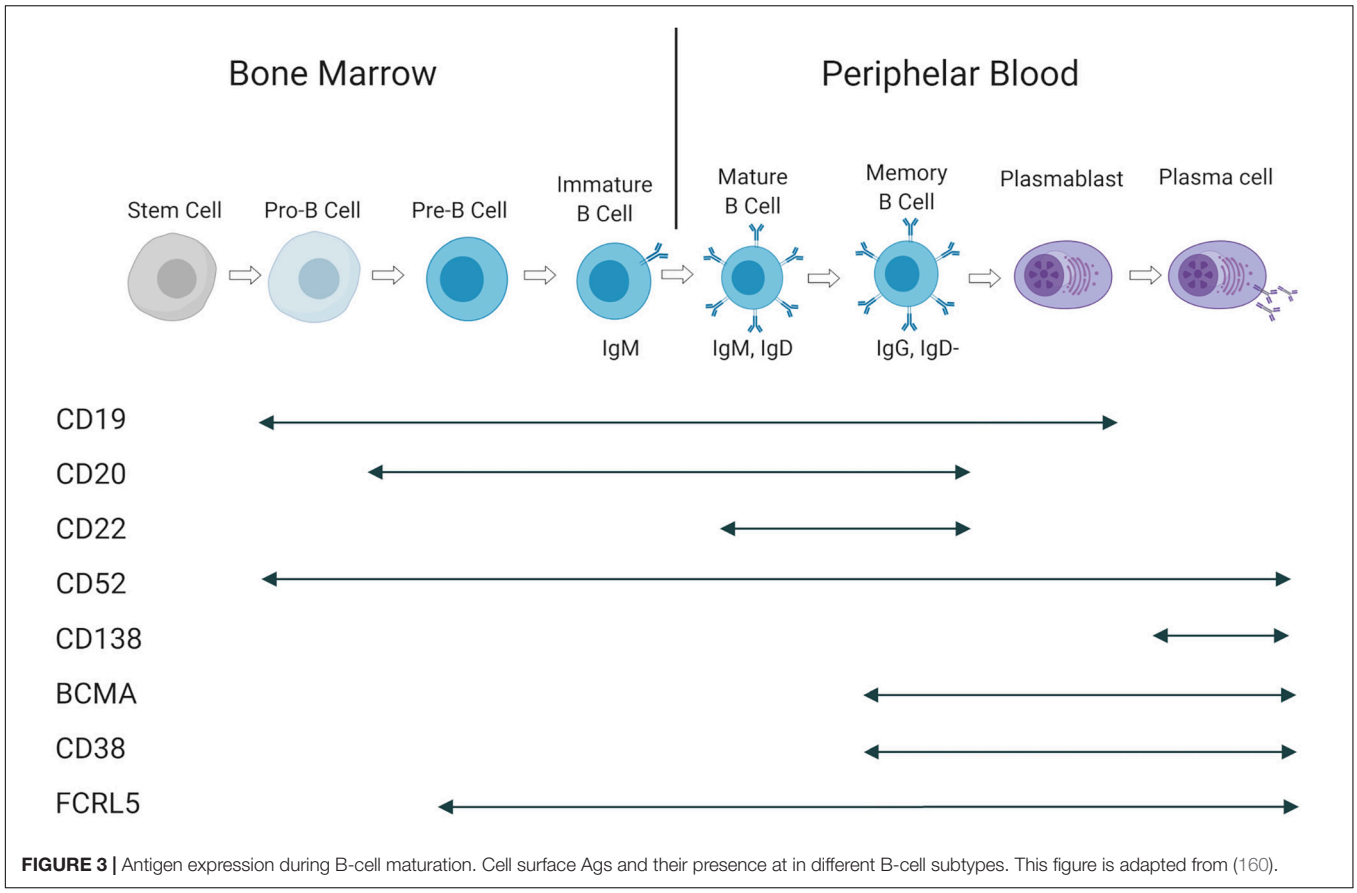

FIGURE 3 | Antigen expression during B-cell maturation. Cell surface Ags and their presence at in different B-cell subtypes. This figure is adapted from (160).

traditional approaches (75). The Ags expressed during B-cell development are illustrated in **Figure 3**. The clinical development of blinatumumab will be discussed later. CD19-binding CAR-T cells were recently approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of relapsed ALL and aggressive NHL.

BISPECIFIC ANTIBODIES IN CLINICAL DEVELOPMENT

A selection of BsAbs in clinical development is shown in **Table 3**.

Clinical Development for ALL, CLL, and/or NHL (CD19 – CD3)

CD19 is expressed from the early development of B-cells up to their differentiation into plasma cells. Targeting CD19 results in B-cell aplasia, which is considered as manageable since patients can receive intravenous Igs until the recovery of the B-cell lineage. When compared with other B-cell Ags, its broad expression profile and low negative regulation rate (76) makes CD19 a suitable target for B-cell malignancies. It is expressed in 80% of ALL cases, 88% of B-cell NHL and all cases of CLL (77).

Three main (anti-CD19 × CD3) BsAbs have been developed for the treatment of B-cell ALL: Blinatumumab, AMG103 (BiTE), MGD011 (dual-affinity re-targeting Ab: DART) and AFM11 (Tandem diabody: TandAb).

BiTE Anti-CD19 – CD3 (Blinatumomab; AMG103)

Blinatumomab is a BiTE with excellent cell-binding capacities due to its small size allowing a better tumor penetration compared to Igs (78). In humans, it was initially explored in relapsed/refractory (r/r) NHL and afterwards in ALL (79). It was approved by the FDA in December 2014 and the EMA in December 2015 for the treatment of r/r Ph-negative ALL (23, 80–83). However, it is currently being tested in clinical trials for other hematologic malignancies, such as NHL and MM.

Given its short half-life, blinatumomab is continuously administrated via an intravenous infusion, at a constant rate (after an increase in the initial dose) and by repeated cycles of 4 weeks, that are interrupted with 2 weeks without treatment (23). The observed side effects are mostly mild to moderate and occur during the first cycle. The treatment generally starts under vigilant monitoring with a lower dose during the first 7 days. The most commonly observed adverse effects are chills, pyrexia, constitutional symptoms and reversible neurological events, such as tremors, seizures, aphasia, and ataxia. Furthermore, up to 70% of patients had symptoms of a transient CRS (84). In order to minimize these effects, premedication with dexamethasone is required on the first day of each cycle and on the first day of any dose increase (5, 23).

TABLE 4 | Clinical trials of BsAbs in combination with different immunotherapeutic strategies (selected trials).

Names (Sponsors)	Targets	Diseases indication	Phase (NCT#)
Combinations with immune modulators			
Combination of Blinatumomab and Nivolumab (anti-PD-1 mAb) +/− Ipilimumab (anti-CTLA4 mAb) [National Cancer Institute (NCI)]	CD3 × CD19 × PD-1 (x CTLA4)	B-ALL	Phase I (NCT02879695)
Combination of Blinatumomab and Pembrolizumab (anti-PD-1 mAb) (Merck Sharp & Dohme Corp., Amgen)	CD3 × CD19 × PD-1	B-ALL	Phase I/II (NCT03160079)
Combination of Blinatumomab and Pembrolizumab (anti-PD-1 mAb) (Amgen)	CD3 × CD19 × PD-1	NHL	Phase I (NCT03340766)
Combination of Blinatumomab and (anti-PD-1 mAb) (City of Hope Medical Center)	CD3 × CD19 × PD-1	ALL	Phase I/II (NCT03512405)
Combination of Blinatumomab and Pembrolizumab (anti-PD-1 mAb) (Children's Hospital Medical Center, Cincinnati)	CD3 × CD19 × PD-1	B-cell lymphoma and leukemia	Phase I (NCT03605589)
Combination of BTCT4465A and Atezolimumab (anti-PD-L1 mAb) (Genentech)	CD3 × CD20 × PD-L1	CLL, NHL	Phase I (NCT02500407)
Combination of REGN1979 and REGN2810 (cemiplimab: anti-PD-1 mAb) (Regeneron Pharmaceuticals)	CD3 × CD20 × PD-1	Lymphoma	Phase I (NCT02651662)
Combination of REGN1979 and REGN2810 (anti-PD-L1 mAb) (Hoffmann-La Roche)	CD3 × CD20 × PD-L1	NHL	Phase I (NCT03533283)
Combination with mAb			
Combination of JNJ-64407564/JNJ-64007957 and Daratumumab (Janssen)	CD3 × BCMA or GPRC5D × CD38	MM	Phase I (NCT04108195)
Combination with ADC			
Combination of BTCT4465A and Polatuzumab vedotin (anti-CD79b × MMAE) (Hoffmann-La Roche)	CD3 × CD20 × ADC	B-cell NHL	Phase I (NCT03671018)

Data available as of November 05, 2019. Molecules are classified based on target antigens. ADC, antibody-drug conjugate; BCMA, B-cell maturation antigen; CTLA4, cytotoxic T-lymphocyte-associated protein 4; GPRC5D, G protein-coupled receptor family C group 5 member D; mAb, monoclonal antibody; MMAE, monomethyl auristatin E; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand.

Blinatumomab is currently in Phase I and II clinical trials in combination with monoclonal Abs (mAbs) targeting inhibitory checkpoints, such as programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) (**Table 4**).

DART CD19 – CD3 (MGD011)

MGD011 (duvortuxizumab) is a CD19 × CD3 DART with a silenced, human IgG1 Fc domain. The presence of this Fc domain prolongs its circulating half-life (approximately 14.3 to 20.6 days), similar to conventional mAbs, allowing for an administration every 2 weeks (85). The humanized Ab arms have a 10-fold greater affinity for CD19 than for CD3, thereby enabling preferential binding to target cells, while minimizing the engagement of CD3 in the absence of target cells. Although the preclinical results in murine NHL models was promising, the clinical development of MGD011 was discontinued early due to high levels of neurotoxicity observed in a Phase I study on the treatment of B-cell malignancies (NHL, CLL, and NCT02743546) (85).

TandAb CD19 – CD3 (AFM11)

AFM11 is a tetravalent bispecific TandAb with two binding sites for CD3 and two for CD19. This structure increases the binding affinities for CD19 and CD3 by approximately 5- and 100-fold, respectively, compared to those of BiTE. Furthermore, AFM11 potency is not correlated with CD19 density on the surface of the target cell (86). This BsAb was tested in phase I studies for the treatment of ALL (NCT02848911) and r/r NHL (NCT02106091). These two clinical trials were suspended due to neurological side effects that caused the death of one patient and life-threatening toxicities in two others. Therefore, the risk/benefit profile was not favorable with the dosing regimens studied, putting an end to these two clinical studies.

Clinical Development for ALL, CLL, and/or NHL (CD20 – CD3)

The CD20 Ag is expressed exclusively on mature B-cells and not on B-cell precursors, stem cells and plasma cells. It is also observed on the surface of malignant B-cells: more than 95% of B-cells in NHL and other B-cell malignancies express CD20.

TriFab CD20-CD3 (FBTA05)

FBTA05 (Lymphomun) has a TriFAb format; the third functional site is the Fc region which provides an additional capacity to recruit accessory cells bearing the Fcγ receptor (FcγR) (macrophages, dendritic cells, NK-cells and neutrophil granulocytes) (87). Promising responses have already been observed in pediatric patients (88, 89), but details on its further development or its current status are not clear.

IgG4-Based CD20 – CD3 (REGN1979)

REGN1979 is a fully humanized bispecific IgG4 Ab designed to resemble natural human Abs (90). As a result, this construct has the advantages of native Abs, such as stability, low aggregation propensity, low immunogenicity and good pharmacokinetics. This BsAb induces prolonged B-cell depletion in the peripheral blood as well as in lymphoid organs in preclinical models (90). In a phase I study on r/r NHL, administration of BsAb resulted in an overall response of 100% in follicular lymphoma and provided a complete response in two patients who did not respond to CAR T-cell therapy (91).

IgG1-Based CD20 – CD3 (Mosunetuzumab)

Mosunetuzumab (or BTCT4465A) is a another full-length, humanized IgG1 molecule with an almost native Ab structure using KiH technology. The first clinical results with mosunetuzumab were recently reported: In the patients with r/r aggressive NHL, the objective response rate (ORR) was 37.1%, with a complete response rate of 19.4%. Higher response were seen in the group with indolent NHLs with an ORR of 62.7% and complete response (CR) rate of 43.3% (92).

CD20-CD3 (RG6026)

RG6026 is a BsAb that binds to CD20 and CD3 in a 2:1 format, providing better affinity for the tumor Ag. The CD3 binding arm is fused directly to one of the CD20 binding arms via a short flexible linker. RG6026 also has a modified heterodimeric Fc region that prevents binding to FcγRs, while binding to the neonatal Fc Receptor is maintained, which results for an extended circulatory half-life (93). It showed significant *in vitro* and *in vivo* activity even on cells expressing low levels of CD20, it remains active in the presence of competing anti-CD20 antibodies and can potentially bypass the resistance to rituximab (94). Furthermore, its cytotoxicity activity has been observed even at very low effector:target ratios (95).

Clinical trials are underway to evaluate the efficacy of these different anti-CD20 × anti-CD3 BsAbs (**Table 3**). Several of these CD20-targeting BsAbs (Mosunetuzumab, REGN1979, and RG6026, etc.) are currently in Phase I clinical trials in combination with monoclonal Abs targeting the PD-1 inhibitory checkpoint or its ligand, PD-L1 (**Table 4**).

Clinical Development for Lymphoma (CD30 – CD16A)

AFM13 is a tetravalent BsAb in the TandAb format without Fc domain (24). Therefore, it has two binding sites for CD30, located between two binding sites for CD16A. The center of the molecule interacts with the CD30 Ag, whereas the effector cell binds to both ends of the molecule. It is used to direct NK-cell toxicity to CD30-expressing lymphoma cells. It has been shown that AFM13 activates NK-cells only after binding to CD30 (94). AFM13 has shown signs of activity in a Phase I study, as well as effective NK-cell activation and a decrease in soluble CD30. Moreover, it has been well tolerated and may even be better tolerated than T-cell based BsAbs (24). AFM13 is currently in phase II clinical development (**Table 3**).

Clinical Development for MM (CD38 – CD3)

The uniformly overexpressed CD38 Ag is the most widely studied target in the treatment of MM (96). Intriguingly, it is also expressed by many other hematopoietic cells, but treatment with the monoclonal Ab daratumumab is safe and without major side effects (96).

Several humanized anti-CD38/CD3 XmAb BsAbs and with different affinities for CD38 and CD3, were simultaneously evaluated during the preclinical stage. The best *in vitro* and *in vivo* results were obtained with AMG424. Although it has a lower affinity for CD3 to prevent an uncontrolled CRS in the presence of soluble CD38, it shows strong anti-tumor effects (97). Given that CD38 is also expressed by T-cells, a fratricide problem could interfere with the activity of AMG424. A Phase I Study (NCT03445663) evaluating the safety, tolerability, pharmacokinetics, pharmacodynamics, and efficacy of AMG 424 in recurrent/refractory Multiple Myeloma (r/r MM) began in 2018 and will end in 2022.

GBR 1342 is another anti-CD38/CD3 BsAb that is developed by Glenmark. It contains a complete Fc domain with a reduced effector function. In preclinical studies, GBR1342 showed a more potent anti-cancer effect than the anti-CD38 mAb daratumumab. It efficiently recruited T-cells and induced CD38 + cell depletion in the blood and especially the bone marrow (98). A Phase I study (NCT03309111) started in October 2017 evaluating the safety and tolerability of GBR 1342.

Clinical Development for MM (BCMA – CD3)

BCMA is a membrane Ag expressed by malignant plasma cells as well as plasmacytoid dendritic cells. In contrast, it is not expressed on naive B-cells, CD34 + hematopoietic cells or any other normal tissue cells (99–101). BCMA has several advantages making it a highly studied target as part of the treatment for MM. First, BCMA is highly expressed by MM cells, as well as in patients with poor prognosis. Second, a rapid re-emergence of B-cell immunity after the end of the anti-BCMA treatment would be possible since this Ag is not expressed early in B-cell development. Third, the lack of BCMA expression in other bone marrow populations prevents off-tumor toxicities.

Several BsAbs are currently in clinical trials to evaluate their efficacy primarily in patients with advanced MM who have relapsed or are refractory to standard treatment (**Table 3**).

BCMA-CD3 BiTEs (AMG420 and 701)

AMG420 (or BI 836909) is a BiTE that has a short half-life time and therefore must be administered intravenously for 4 weeks followed by 2 weeks treatment-free. While AMG420 induces potent lysis of BCMA-positive MM cells *in vitro* and *in vivo*, BCMA-negative cells were not affected. Accordingly, clinical trials started for the treatment of r/r MM in 2015 (NCT02514239) and in 2019 (NCT03836053) (102). In a phase I study including 42 refractory MM patients, a high response rate of 70% was observed including 50% MRD-negative complete responses. The most common side effects were infections and

polyneuropathy. AMG701 is a half-life–extended BiTE that contains the single-chain variable fragments of AMG420. It is suitable for once-weekly dosing and is currently tested in a phase I trial. Comparison of the observed responses and toxicities, allows to study the clinical implications of such a half-life extension.

BCMA-CD3 CrossMabs (EM801, CC-93269)

EM801 is a CrossMab in the 2 + 1 format. Its prolonged half-life due to maintenance of the Fc region allows for a convenient weekly intravenous treatment. Nonetheless, it is eliminated from the circulatory system within 1 to 2 months of treatment discontinuation. EM801 achieved lysis of 90% of myeloma cells after 48 h with a very low E:T ratio (103). The first results of a related molecule, EM901/CC-93269 (ENgMab/Celgene), on 30 r/r MM patients were recently presented: clinical activity was seen at higher doses of the drug with almost 90% of the patients responding at the highest dose. 76% of patients developed a CRS which was severe (> Grade 3) in one patient (104).

IgG2a-Based BCMA-CD3 (PF-06863135)

PF-06863135 (PF-3135) is a humanized BsAb using a IgG2a backbone with mutations in the Fc part that promote heavy chain heterodimer formation and reduce Fcγ receptor binding (105). This BsAb showed potent anti-myeloma activity in both *in vitro* and *in vivo* models and its toxicity profile in cynomolgus monkeys was acceptable (105). PF-06863135 is currently undergoing a Phase I study to assess its safety and tolerability (NCT03269136) (106).

Clinical Development for MM (FcRL5 – CD3 and GPRC5D – CD3)

Two new targets have recently emerged as part of the MM-related targets: Fc Receptor-Like 5 (FcRL5) and G-protein coupled receptor family C group 5 member D (GPRC5D).

The first (also known as FcRH5, IRTA2, or CD307) is a specific and exclusive surface marker of the B-cell lineage. Its expression is detected starting from the pre-B-cell stage (107). However, unlike other B-cell-specific surface proteins, FcRL5 expression is preserved in normal and malignant B-cells (including plasma cells). This suggests a potential broader applicability of this target in B-cell malignancies, such as chronic lymphocytic leukemia, mantle cell lymphoma, diffuse large B-cell lymphoma, and follicular lymphoma (107, 108).

In contrast, GPRC5D is expressed on the surface of malignant cells involved in multiple myeloma without being expressed at appreciable levels by normal hematopoietic cells, such as T-cells, NK-cells, monocytes, granulocytes and bone marrow progenitors, including hematopoietic stem cells (109). High mRNA expression of GPRC5D was observed in patients with MM, whereas only low expression was detected in normal tissues. Its mRNA expression was also significantly correlated with poor overall survival rates (110). As a result, its very limited expression profile makes it a suitable target in MM treatment.

Two BsAbs have been developed against these two targets and are currently in a phase I clinical trial: RG6160 which targets FcRL5 (NCT03275103) and the DuoBody JNJ-64407564 which targets GPRC5D (NCT03399799) (**Table 3**). Both showed *in vitro* and *in vivo* B-cell depletion and tumor growth suppression in myeloma models (35, 109).

CONCERNS IN CLINICAL DEVELOPMENT

Cytokine Release Syndrome (CRS)

CRS is a potentially fatal systemic inflammatory reaction that is observed after the infusion of immunotherapeutic agents (monoclonal Abs, BsAbs, and CARs). Although our understanding of CRS is incomplete, different immune populations including T-lymphocytes, monocytes and macrophages are activated, all resulting in a mass production of inflammatory cytokines, particularly interleukin (IL)-6 and interferon (IFN)-γ (111). Although the immunological cascade is initiated by T-cell activation, this massive systemic production of toxic cytokines is mainly due to monocyte and macrophage activation. T-cell IFN-γ, macrophage IL-6, IL-10 and tumor necrosis factor alpha (TNF-α) seem to cooperate to facilitate this cytokine release (112). In addition, IL-6 has been shown to play a central role in humans and mice in the development of CRS (111, 113). Patients presenting CRS usually develop mild fatigue, fever, chills, headache, arthralgia, or even more serious life-threatening problems, such as hypotension, tachycardia, vascular leaks and circulatory collapse during or immediately following administration of the drug.

In general, signs and symptoms of CRS only appear during the first cycle of the drug, and not later during subsequent administrations. This CRS is not implicated in the mechanisms of action of T-cell directed immunotherapies (114), as the response to treatment is unaffected by the severity of CRS (115). A mitigation strategy based on corticosteroids and IL-6 blockade has been proposed to minimize the release of toxic cytokines (112).

An alternative way to avoid CRS-related problems is to dissociate tumor cell destruction and cytokine release. There are two distinct thresholds for T-cell activation based on the number of TCR- peptide-MHC (pMHC) complexes formed (116). The formation of two TCR-pMHC complexes is sufficient between a T-cell and an Ag-presenting cell, to trigger T-cell-mediated cell lysis. On the other hand, 10 TCR-pMHC complexes are required for the formation of a complete immune synapse and cytokine secretion. Thus, adjusting the binding characteristics for the CD3-binding arm, a BsAb could more closely mimic the natural TCR-pMHC induced T-cell activation (117). Consequently, new CD3-binding Abs have been generated that bind to multiple epitopes on CD3 with a wide range of affinities and agonist activities. Functional studies were realized with BsAbs that integrated the different CD3-binding domains. A BsAb with a new T-cell-engaging domain could be created that elicited strong *in vivo* tumor cell killing and low levels of cytokine release (118).

Neurotoxicity

Neurotoxicity is the second most common adverse effect observed with different BsAbs. Symptoms may range from subtle changes in personality to tremors, vertigo, confusion,

and focal neurological symptoms to more serious episodes of encephalopathy, ataxia, cerebellar alteration, convulsions and delirium (23). The pathophysiology of these neurotoxic effects still has not been determined but, as in CRS, inflammatory cytokines appear to be involved (119).

Grade 3 or higher neurotoxicity occurs in approximately 10 to 20% of the patients treated with blinatumomab (5, 120). However, in most cases, the neurological side effects were reversible after stopping the BsAb perfusion and initiation of corticosteroids. Furthermore, grade 3 or higher neurological events were avoided using a progressive dosing regimen and the prophylactic administration of dexamethasone. Although the application of steroids relieves the central nervous system symptoms, it could potentially hamper the immune response. While reduced levels of inflammatory cytokines were produced by dexamethasone-treated T-cells, there was no inhibitory effect of dexamethasone on the cytotoxic capacities of T-cells observed (121). This indicates that dexamethasone does not interfere with the therapeutic efficacy of BsAbs.

Administration Route

The most commonly used administration route for BsAbs is intravenous (IV) perfusion. Although it has advantages in terms of pharmacokinetics and pharmacodynamics, it has certain drawbacks with regards to patient convenience, access to therapeutic targets and cost of treatment. The reduced half-life time of some BsAbs results in either more frequent administrations or continuous infusion (11, 12). On the other hand, the addition of an Fc domain facilitates the BsAb purification, improves solubility and stability, and molecule's half-life (12). However, although BsAbs with an extended half-life may ease the logistics of administration, prolonged exposure could potentially increase the toxicity. Ongoing clinical trials will test this hypothesis and confirm or refute it.

Resistance Mechanisms

T-Cell Exhaustion/Dysfunction

During cancer development, T-cells rapidly become dysfunctional due to persistent Ag-exposure. This reduces their proliferation capacity and their cytotoxic effector function. Moreover, several inhibitory receptors (such as PD-1, CTLA-4, T-cell immunoglobulin and mucin domain-3 (TIM-3), Lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and ITIM domain (TIGIT) are overexpressed by malignant cells (122, 123). Among them, the PD1/PD-L1 axis appears to be a central process in T-cell dysfunction (124). Targeting these inhibitory pathways is currently used to block immune suppressive signals coming from tumor cells and to prolong T-cell activation.

T-cell exhaustion is characterized by a progressive loss of function, such as proliferation, cytokine production, and cell lysis. T-cells do not become totally inactive, but fail to effectively eradicate cancer cells. Three distinct signals are normally required for optimal T-cell activation and proliferation. First, an Ag recognition via the TCRs is needed, followed by a costimulation and a cytokine release by the T-cells, which is required for their expansion. BsAb only provide the first signal. However, BiTEs and many other Ab formats may trigger the formation of an effective immunological synapse, abolishing the need for co-stimulation (125). Co-activation of T-cells through CD28 or 4-1BB, will increase the activation of T-cells by BsAbs (126, 127). Regarding the third requirement, new BsAb constructs have been developed to include cytokine IL-15 (128). Moreover, as mentioned previously, the blockade of PD-1 or its ligand, PD-L1, can successfully reactivate T-cell function.

Unfortunately, most patients do not maintain sustainable responses to this treatment. The lack of a sustainable response can be at least partly explained by the presence of other inhibitory pathways in T-cells. Thus, the identification of resistance and evasion mechanisms as well as the understanding of the processes that direct and maintain the various dysfunctional T-cell states are still a major concern for enabling effective BsAb activity targeting T-cells, while avoiding potentially life-threatening autoimmune side effects (129).

Antigen Escape

Tumor cells can also downregulate a targeted Ag and circumvent immune recognition during treatment. For example, loss of CD19 has been observed in patients with ALL, contributing to progression of the leukemia in 10 to 20% of cases. Altered membrane traffic and export (130) as well as, acquired mutations and alternative splicing explain this loss of expression at the cell-surface, while its intracellular abundance is preserved (131). Alternative splicing can, for example, result in the loss of CD19 extracellular domain (131). This leads to a conformational change in the extracellular domain of CD19, while the loss of a chaperone molecule (CD81) can lead to the intracellular accumulation of CD19 (130).

Consequently, a potential strategy to control Ag escape is to combine the targeting of several Ags in order to generate T lymphocytes that can recognize several Ags expressed on the tumor cells. For instance, a clinical study evaluating the efficacy of an anti-CD19/anti-CD22 BsAb is currently ongoing (NCT02370160) (132) (**Table 3**).

Immunosuppressive Microenvironment

Another major concern is the possible involvement of tumor microenvironment factors, such as immunosuppressive regulatory T lymphocytes (Tregs). Given that BsAbs trigger T-cell activation via binding to the CD3 complex, other T lymphocyte cell subtypes, besides effector T lymphocytes, will also be activated (133). A high percentage of Tregs present in the tumor environment predicts a resistance to treatment. For example, Tregs, activated by blinatumomab, are able to suppress the proliferation of effector T-cells and the subsequent cell lysis. As a result, T-cell depletion prior to administration of blinatumomab may increase effectiveness for non-responding patients treated with blinatumomab (19).

Immune Checkpoint Receptor PD-1

PD-1 is a co-inhibitory receptor that acts as an immune checkpoint. It is used to attenuate immune responses by limiting the duration and intensity of the immune reaction. Tumor cells often express its ligand, PD-L1, to evade immune system

attacks (134). It is an adaptive mechanism of immune escape in response to pro-inflammatory cytokines (135). A wide range of anti-PD-1 antibodies (nivolumab, pembrolizumab) or anti-PD-L1 antibodies (atezolizumab, durvalumab, avelumab) have been tested in mono- or in combination therapy (136). However, PD-L1 is widely expressed on healthy tissues and therefore, the efficacy of these blocking Abs can be reduced due to binding to PD-L1 positive normal cells. This may lead to blind activation of T-cells, including those involved in (auto)immune-related adverse events such as endocrinopathy (for example, thyroiditis), dermatitis, pneumonia, hepatitis, and colitis (137–139).

Immune modulation through PD-1 is one of the mechanisms of resistance to blinatumomab (140). While refractory leukemic blasts overexpressed PD-L1, T-cell exhaustion was observed with overexpression of PD-1. Combination of blinatumomab and the anti-PD-1 antibody Pembrolizumab enhanced T-cell function and induced an anti-leukemic response in a 12-year-old patient with refractory ALL (140). The activity of blinatumomab could also be restored by adding an anti-PD-L1 × CD28 BsAb that abolished the PD-L1 mediated resistance and even reverted the negative PD-L1 signaling into positive costimulation through CD28 on T-cells (141). The combined action of PD-1/PD-L1 blocking Abs and BsAbs inspired the design and initiation of clinical studies combining blinatumomab with checkpoint inhibition as summarized in **Table 4**. In order to improve the clinical benefit, BsAbs that simultaneously target two immune checkpoints have been developed. For example, the dual blockade of PD-1 and LAG-3 with monoclonal Abs further suppresses T-cell activation. For instance, an anti-PD-1/anti-LAG-3 DART, called MGD013, binds specifically to both PD-1 and LAG-3 (142). Blocking both pathways enhanced T-cell responses compared to those observed upon independent blockade of either the PD-1 or LAG-3 pathways alone. The BsAb KN046 is another that binds to PD-L1 on the tumor cells and to CTLA-4 expressed by the T-cells. However, the increase in anti-tumor activity has been associated with a significant increase in the number of adverse events due to over-activation of the immune system. Consequently, a new approach is currently being investigated. It consists in the deletion of the PD-1 pathway via high-affinity PD-1 binding, while inhibiting CTLA-4 with a low affinity binding arm. This construct inhibits CTLA-4 in double-positive T-cells while reducing the binding to peripheral T lymphocytes expressing CTLA-4, resulting in better tolerability (143).

The Co-stimulatory Receptor 4-1BB

4-1BB (CD137) is a potent co-stimulatory receptor that is upregulated on effector T lymphocytes including tumor infiltrating T-cells. Its stimulation improves cytotoxic function, as well as the induction of an immunological memory (144). In addition to its function on T-cells, it has been shown to improve the cytotoxic function of NK-cells (145). 4-1BB-binding monoclonal Abs are classified according to their agonistic capacities and Fc receptor affinities. While urelumab is a strong agonist and inducing signal activation without Fc receptor binding, the basal agonistic activity of utomilumab is weak but increases after Fc receptor crosslinking (146). The clinical development of these first-generation Abs was stopped: utomilulab showed only a reduced efficacy (although no major toxicities were seen) and urelumab showed efficacy but also severe liver toxicity (147, 148). Interestingly, new 4-1BB binding Abs have recently been created by adapting the level of intrinsic agonistic activity, the FcγR interactions, the IgG subclass and Ab affinities (146, 149). Another strategy to overcome the limitations of the first- generation Abs is the integration of 4-1BB-binding domains in BsAbs.

A few BsAbs containing a tumor Ag-binding fragment and a 4-1BB agonist have been developed (150–152). The main characteristic of these compounds is the lack of significant 4-1BB activation in the absence of tumor Ag binding, ensuring tumor-localized immune activation. For example, a BsAb that simultaneously targets 4-1BB and the CD19 tumor Ag was developed for systemic administration (153). Since additional mutations in the Fc region prevents Fcγ receptor cross-linking, the 4-1BB in this construct is only activated when cross-linked to CD19 and thus, hepatic toxicity is avoided (9). Another example of BsAb targeting checkpoint agonists is INBRX-105 (Inhibrx) which is directed toward PD-L1 and 4-1BB. While simultaneously suppressing inhibition via the PD-1 – PD-L1 axis, it is designed to only activate T-cells via 4-1BB in the tumor environment when it encounters PD-L1 (17).

Immune Checkpoint Receptor CD47

CD47 [Integrin-associated protein (IAP)] is ubiquitously expressed in normal tissues and can be found on mesenchymal stromal cells and blood cells, particularly erythrocytes and platelets, and is generally upregulated in cancers. When it binds to its ligand, the signal regulatory protein α (SIRPα) which is an inhibitory receptor on macrophages and dendritic cells, CD47 sends "don't eat me" signals by inhibiting phagocytosis of tumor cells and triggering an immune evasion (154).

Hematological cancer cells overexpress CD47 in order to evade removal by phagocytes (macrophages and dendritic cells) (154, 155). As a consequence, both the innate and adaptive anti-cancer immune responses are suppressed. Therefore, CD47 neutralizing antibodies could improve tumor lysis by effector cells. However, CD47 is also widely expressed on normal cells (156). Thus, a general blockade of the CD47/SIRPα interaction may result in the removal of normal healthy cells and may be associated with toxicity.

Furthermore, the abundant expression of CD47 throughout the entire human body could eventually lead to the formation of "Ag sinks" that would prevent anti-CD47 antibodies from reaching the targeted tumor. To circumvent this problem, BsAbs with a low affinity for CD47 and a high affinity for a tumor Ag have been developed, which guarantee CD47 to be bound by BsAb only on tumor cells co-expressing both Ags. For example, a CD47 × CD19 BsAb (TG-1801, NI-1701, NovImmune, TG Therapeutics) induced increased phagocytosis by Fc and retained its activity in the presence of high amounts of non-tumor-associated CD47 (157). However, the functional Fc domains present in this BsAb can cause the off-target premature activation of Fc receptor (FcR)-expressing phagocytes, thereby causing systemic toxicity. Another BsAb format called

RTX-CD47, targeting CD47 and CD20 without an Fc domain, triggered a significant phagocytic removal of both CD20 and CD47 malignant B-cells, but not cells expressing CD47 alone, while preventing toxicity associated with the presence of an Fc domain (158).

CONCLUSION

As seen in different clinical trials, BsAbs are promising tools for the treatment of hematologic B-cell malignancies. They enable different mechanisms of action, each having its own advantages and disadvantages. Although anti-tumor effects are observed, their clinical translation is hampered by limiting side-effects, such as off-target effects, a reduced E:T ratio in pretreated patients, and pharmacological limitations. Therefore, combined expertise in immunology, pharmacology and Ab engineering is required to improve their efficacy. A number of approaches are currently being studied and include combinations with checkpoint inhibitors, chemotherapy and other existing treatments. The different platforms on which BsAbs are produced will further improve their anti-tumor activity. Looking at the variety of targets, indications, mechanisms of action and implicated companies, it is clear that BsAbs will become key players in the field of immunotherapy.

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JC and ML contributed to the conception and design of this review. ML and MK wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Combination of T-Cell Bispecific Antibodies With PD-L1 Checkpoint Inhibition Elicits Superior Anti-Tumor Activity

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T-cell Bispecific Antibodies (TCBs) elicit anti-tumor responses by cross-linking T-cells to tumor cells and mediate polyclonal T-cell expansion that is independent of T-cell receptor specificity. TCBs thus offer great promise for patients who lack antigen-specific T-cells or have non-inflamed tumors, which are parameters known to limit the response of checkpoint inhibitors. The current study deepens the understanding of TCB mode of action and elaborates on one of the adaptive resistance mechanisms following its treatment *in vivo* in humanized mice and syngeneic pre-clinical tumor models. Single-agent TCB treatment reduced tumor growth compared with controls and led to a 2–10-fold increase in tumor-infiltrating T-cells, regardless of the baseline tumor immune cell infiltration. TCB treatment strongly induced the secretion of CXCL10 and increased the frequency of intra-tumor CXCR3+ T-cells pointing to the potential role of the CXCL10-CXCR3 pathway as one of the mechanisms for T-cell recruitment to tumors upon TCB treatment. Tumor-infiltrating T-cells displayed a highly activated and proliferating phenotype, resulting in the generation of a highly inflamed tumor microenvironment. A molecular signature of TCB treatment was determined (CD8, PD-1, MIP-a, CXCL10, CXCL13) to identify parameters that most robustly characterize TCB activity. Parallel to T-cell activation, TCB treatment also led to a clear upregulation of PD-1 on T-cells and PD-L1 on tumor cells and T-cells. Combining TCB treatment with anti-PD-L1 blocking antibody improved anti-tumor efficacy compared to either agent given as monotherapy, increasing the frequency of intra-tumoral T-cells. Together, the data of the current study expand our knowledge of the molecular and cellular features associated with TCB activity and provide evidence that the PD-1/PD-L1 axis is one of the adaptive resistance mechanisms associated with TCB activity. This mechanism can be managed by the combination of TCB with anti-PD-L1 blocking antibody translating into more efficacious anti-tumor activity and prolonged control of the tumor outgrowth. The elucidation of additional resistance mechanisms beyond the PD-1/PD-L1 axis will constitute an

important milestone for our understanding of factors determining tumor escape and deepening of TCB anti-tumor responses in both solid tumors and hematological disorders.

Keywords: solid tumors, immunotherapy, T-cell bispecific antibody, carcinoembryonic antigen T-cell bispecific antibody, programmed death–ligand 1, combination, humanized mice

INTRODUCTION

Targeting T-cells with antibodies that directly enhance T-cell activity, including the checkpoint inhibitory molecules (CPIs) programmed death receptor 1 (PD-1), PD-ligand 1 (PD-L1), and cytotoxic lymphocyte activated antigen 4 (CTLA4) has become an established approach in clinical practice (1–3). Antibodies to checkpoint molecules have gained broad approval in various tumor indications for the treatment of advanced cancer types such as metastatic melanoma, advanced non-small cell lung cancer, or renal cell carcinoma (4). However, despite these advances, obstacles still exist including the inability to predict treatment efficacy and patient response, the need for additional biomarkers, the development of primary and secondary resistance to cancer immunotherapies, the lack of clinical study designs that are optimized to determine efficacy and toxicity (and their relationship), and high treatment costs (5).

T-cell Bispecific Antibodies (TCBs) elicit anti-tumor responses by cross-linking of T-cells to target tumor cells (6, 7). TCB-mediated polyclonal T-cell activation is independent of the T-cell receptor specificity and does not require (at least initially) costimulatory signals. Thus, factors normally affecting the efficiency of CPIs to mount an endogenous anti-tumor immune response, including MHC downregulation, antigen presentation, the frequency of antigen-specific T-cells, T-cell receptor affinity, and T-cell avidity, are less relevant for TCB activity. Hence, TCBs are a highly attractive approach to activate T-cells regardless of their antigen specificity. Due to the increase of intra-tumor T-cell infiltration upon treatment (8–10), TCBs offer great promise in patients that lack the baseline antigen-specific T-cells (or any type of T-cells, the so called immune desert tumors), which is thought to render responses to checkpoint inhibition less likely (5).

Although more than forty different bispecific antibodies have been described to date (6, 11–14), the promising results obtained in preclinical studies do not translate directly into the clinical setting. Only two TCBs were approved for use in the clinic so far: catumaxomab and blinatumomab. Catumaxomab targets epithelial cell adhesion molecule (EpCAM) and was initially approved by the European Medicines Agency (EMA) in 2009 for the treatment of malignant ovarian ascites (15), Catumaxomab has not been marketed in the EU since 2014 and market authorization was withdrawn in 2017. Blinatumomab targets CD19 and was approved by the FDA and EMA in 2014 and 2015, respectively, for the treatment of Philadelphia chromosome negative B cell acute lymphoblastic leukemia (16). Promising clinical activity has been reported with other TCBs, particularly in hematological malignancies (6, 7, 17).

Clinical development of TCBs in solid tumors has been challenging and may be hampered by multiple constraints. These include the lack of tumor-specific antigens that are not expressed in primary epithelium (18), the local suppressive tumor microenvironment [characterized by expression of IL10, TGFβ, IDO, COX-2, adenosine, and arginase, and presence of regulatory T-cells and myeloid-derived suppressor cells (19, 20)], and the physical barriers that trap immune cells in the stroma [also called immune exclusion, (21, 22)]. These factors may limit the frequency and activation of effector cells within the tumor (5, 23). Moreover, a dysfunctional T-cell state characterized by the abundance of intra-tumoral PD-1^{hi} T-cells hampered TCB activity *ex vivo* (24), providing an additional primary resistance mechanism affecting TCB activity.

TCB-induced T-cell activation is has been shown to upregulate PD-1 expression on T-cells and induce PD-L1 expression on tumor cells (IFNγ driven) (8, 9, 25–29). This may lead to adaptive immune resistance mechanisms related to the TCB mode of action, similar to what has been described for checkpoint inhibition (30, 31). The same studies provided pre-clinical evidence that blockade of the PD-1/PD-L1 axis restores TCB activity *in vitro* and *in vivo* and provided the rationale for combining TCBs with therapeutic strategies targeting T-cell dysfunction in the clinic to potentiate the activity of TCBs (13, 32). These studies led to several Phase 1 trials evaluating T-cell engaging bispecific antibodies in combination with checkpoint inhibitors, particularly anti-PD-1/PD-L1 antibodies (6, 7, 11).

We have previously described the so-called 2:1 TCBs that carry two tumor antigen binding moieties and a single CD3 binding moiety in an IgG-based format (33, 34). This 2:1 format shows advantageous properties over classical 1:1 TCBs (9). In the current study, we deepen the understanding of TCB mode of action by characterizing molecular and cellular features of immune cells and tumors following TCB treatment *in vivo* in humanized mice and syngeneic tumor models, and provide additional evidence that combination with checkpoint inhibitors of the PD-1/PD-L1 axis improves TCB activity. We demonstrate that combination treatment increases the frequency of total intra-tumor T-cells, and identify the CXCL10-CXCR3 pathway as one of the potential mechanisms mediating such increase. We also show that combination treatment lowers the intra-tumor frequency of putatively exhausted T-cells. Together, the study corroborates the relevance of blocking the PD-1/PD-L1 axis to improve TCB activity and highlights the importance of exploring additional combinations that enable generation of T-cells maintaining the optimal functional status.

MATERIALS AND METHODS

Therapeutic Antibodies

The human carcinoembryonic antigen TCB (CEA-TCB; cibisatamab) monoclonal antibody was generated as described previously (8). A murine surrogate of CEA-TCB (muCEA-TCB) was generated for studies in fully immunocompetent mice on a fully silent murine IgG_1 backbone. MuCEA-TCB antibody was generated using an anti-CEA binder that binds to a partially overlapping (but not competing) epitope to the human CEA binder include in CEA-TCB antibody and contains the murine-specific anti-CD3 binder (clone 2C11). The potency of muCEA-TCB is about 10-fold lower than the potency of human CEA-TCB, attributed to the lower activity of the murine anti-CD3 antibody clone and putatively to the lower cytotoxic activity of murine splenocytes in *ex vivo* killing assays used to profile the activity of the surrogate molecule (data not shown). The anti-PD-L1 monoclonal antibody used in the humanized NOG mouse studies is the clone YW243.55.S70 on a muIgG1 DAPG backbone. The anti-PD-L1 monoclonal antibody used in the immunocompetent mouse studies was mIgG1 anti-PD-L1 antibody (clone 6E11), which reacts to human and murine PD-L1) (35).

Cell Lines

The MKN-45 human gastric adenocarcinoma cell line used in humanized NOG mouse studies was purchased from DSMZ (Braunschweig, Germany; Cat No.: ACC 409). The cells were cultured in DMEM containing 10% FCS and 1% Glutamine and split 1:3 to 1:5 every 2–4 days. The HPAF-II pancreatic adenocarcinoma cell line that was also used in humanized NSG mouse studies was purchased from the American Type Culture Collection (Manassas, VA 20110 USA; Cat No.: CRL-1997). The MC38-hCEA for use in the immunocompetent human CEA transgenic mouse (huCEA Tg mice) study were derived from a mouse colon adenocarcinoma and engineered to express human CEA, obtained from Beckmann research institute of City of Hope (Duarte, CA, USA) (36). The WSU-DLCL2 human diffuse large cell B cell lymphoma (DLBCL) cell line used in the humanized mice studies with CD20-TCB were obtained from the European Collection of Cell Culture. MV3 is a human melanoma cell line, that was established by Ruiter DJ (Department of Pathology, University Hospital Nijmegen, Netherlands) (37). The cells were cultured in DMEM, containing 10% FCS and 1% GlutaMAX and split 1:3 to 1:6 every 3–4 days. Skov3 (ATCC, HTB-77) is a human ovary adenocarcinoma cell line. The cells were cultured in RPMI containing 10% FCS and 1% Glutamine and split 1:4 to 1:8 every 4 days. HT-29 (ATCC, HTB-38) is a human, female Caucasian colon adenocarcinoma cell line. The cells were cultured in McCoy's 5A +10% FCS and 2nM GluMax and split 1:3 to 1:8 every 2–4 days. LS174T (ATCC, CL-188) is a human colon carcinoma cell line. The cells were cultured in in DMEM containing 10% FCS and 1% Glutamine and split 1:3 to 1:5 every 2–4 days.

Mouse Models

All mice were maintained under specific-pathogen-free condition with daily cycles of 12-h light/12-h darkness according to international (Federation of European Laboratory Animal Science Associations) and national [Gesellschaft für Versuchstierkunde/ Society of Laboratory Animal Science (GV-Solas) and Tierschutzgesetz (TierSchG)] guidelines. The study protocol was reviewed and approved by the local government (license ZH193/ 2014). Animals were maintained for 1 week after arrival to get accustomed to the new environment and for observation. Daily continuous health monitoring was conducted.

Hematopoietic stem cell humanized mice were generated in house. Briefly, 4–5-week-old female NOG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}) mice (Taconic, Cologne, Germany) or NOD scid gamma (NSG) mice (Jackson Laboratory, Sacramento, CA USA) were injected i.p. with 15 mg/kg Busulfan (Busilvex, Pierre Fabre Limited) in a total volume of 200 µl. Twenty-four hours later, mice were injected intravenously (i.v.) with 1×10^5 CD34+ cord blood cells (STEMCELL Technologies Inc, Grenoble, France). Fifteen weeks after cell injection, mice were bled and screened for successful humanization by flow cytometry. The generation of these mice will be reported in detail elsewhere.

Immunocompetent human CEA transgenic (huCEA Tg) C57BL/6J mice were obtained under license agreement from Beckmann research institute of City of Hope (36). Double transgenic CEA424-SV40Tag x CEACAM5 Tg mice were obtained under license agreement from LIFE-Center of "Klinikum der Universtität München" (Prof. Dr. Wolgang Zimmermann) (38, 39). Both strains were bred by Charles River Laboratories (Lyon, France).

Subcutaneous Tumor Cell Inoculation

MKN-45 cells, HPAF-II cells and WSU-DLCL2 cells were cultured in RPMI containing 10% FCS (PAA Laboratories, Pasching, Austria) and 1% Glutamax (Gibco, Zug, Switzerland) at 37°C in a water-saturated atmosphere at 5% CO_2. Afterwards, 1×10^6 cells of MKN-45 or HPAF-II cells (1.5×10^6 for WSU-DLCL2 cells) were injected s.c. using a 1:1 mixture of RPMI medium and Matrigel in a total volume of 100 µl.

MC38-huCEA cells were maintained in RPMI medium supplemented with 10% FCS, 500 µg/ml Geneticin (G418, Gibco). Mice were injected s.c. with 0.5×10^6 cells using RPMI medium and Matrigel (1:1) in a total volume of 100 µl.

Therapeutic Antibody Treatment

All mice were injected i.v. or i.p. with 200 µl of the appropriate solution. The mice in the vehicle group were injected i.v. with Histidine buffer (20 mM Histidine, 140 mM NaCl, pH 6.0) and the treatment group with the antibody diluted with Histidine buffer to a volume of 200 µl.

Tumor Volume Measurement

Tumor volume (½ [length × width^2]) was measured 3 times per week by caliper.

Necropsy

At study termination, mice were bled under anesthesia (retro-orbital) and sacrificed. Fresh blood was collected in Heparin tubes. Tumors were surgically removed from all animals and cut into three pieces. One part was snap-frozen in liquid nitrogen for RNA sequencing analysis and multiplex cytokine/chemokine

analysis, one part was fixed overnight in 4% paraformaldehyde for histological analysis, and one part stored in PBS for flow cytometric analysis.

Whole Body SPECT/CT Imaging Technique

CEA-TCB and untargeted TCB (DP47-TCB) antibodies were conjugated with 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (p-SCN-Bz-DOTA; Macrocyclic, Plano, TX, USA) and radiolabeled with ^{111}In and ^{177}Lu, respectively, as described previously (40, 41). Biological and chemical analyses were performed to confirm the integrity of the radiolabeled antibodies.

Female, CD34+ human hematopoietic stem cell engrafted NOD scid gamma (NSG) mice (Jackson Laboratories, Sacramento, CA USA), age ~20 weeks were injected s.c. near the flank of one side with 1×10^6 MKN-45 cells in simple RPMI medium mixed with growth factor reduced Matrigel (1:1 ratio) in 100 µl total injection volume. When tumors reached the target size of 150–300 mm^3, mice were injected with 20 µg of ^{111}In-CEA-TCB and 20 µg of ^{177}Lu-DP47-TCB.

Animals were imaged with standards of ~50 µCi of each pure isotope in an Eppendorf tube placed in the field-of-view underneath the head for spillover coefficient estimation and quantification quality control. At time points of 4, 24, 72, and 120 h post-injection, whole-body, dual-isotope, energy-windowed SPECT scans were acquired, followed by CT for anatomical reference. SPECT acquisition was conducted using energy windows of 162.7–179.9 KeV and 233.1–257.6 KeV for ^{111}In and 107.2–118.5 KeV and 198.0–218.8 KeV for ^{177}Lu. Images were reconstructed, converted to units of µCi, co-registered to corresponding CT images, corrected for crosstalk, and then analyzed using Region of Interest (ROI) based quantification.

Flow Cytometry

Fresh mouse heparin blood (200 µl) was lyzed using the BD Pharm Lyse™ lysing buffer (BD Biosciences, Eysins, Switzerland; Cat No.: 555899) according to manufacturer instructions. Tumors were harvested in sterile PBS and dissociated using the gentleMACS™ system (Miltenyi Biotec, Solothurn, Switzerland). Briefly, tumors were added to C-tubes (Miltenyi Biotec) in a total volume of 5 ml RPMI medium containing Collagenase D solution (Roche, diluted in PBS, final concentration: 1 mg/ml), Dispase II solution (Roche, diluted in PBS, final concentration: 0.64 mg/ml) and DNAse I solution (Roche, diluted in PBS, final concentration: 0.025 mg/ml). After running the tumor program #1, the suspension was incubated for 30 min at 37°C followed by tumor program #2. Cell suspensions were filtered using a BD Falcon™ cell strainer nylon filter (70 µm) and washed twice in FACS buffer (Dulbecco's PBS without Ca^{2+} and without Mg^{2+}, supplemented with 2% FCS and 2 mM EDTA).

Cell suspensions were stained with LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (Life Technologies/ThermoFisher Scientific, Basel, Switzerland) to exclude dead cells according to manufacturer instructions. Afterwards, cells were stained in FACS buffer with anti-human CD45 (Clone: HI30), CD8 (Clone: SK1), CD3 (Clone: UCHT1 or OKT3), CD4 (Clone: OKT4), PD-1 (Clone: EH12.2H7), 4-1BB (Clone: 4B4-1), Ki-67 (Clone: Ki-67), and granzyme B (GZMB) (Clone: GB11), or anti-mouse CD45 (Clone:30-F11), CD3 (Clone: H57-597), CD8 (Clone: YTS156.7.7 or 53-6.7), CD4 (Clone: GK1.5), CXCR3 (Clone: CXCR3-173), FoxP3 (Clone: MF-14), CD62L (Clone: LMEL-14), CD44 (Clone: IM7), PD-1 (Clone: RMP1-30), Tim-3 (Clone RMT3-23), Lag-3 (Clone: C9B7W). All antibodies were obtained from BioLegend/Lucerna-Chem, Luzern, Switzerland except for CXCR3 which was obtained from BD Biosciences. For intracellular staining of Ki-67 and GZMB, first surface staining was performed followed by washing and fixation/permeabilization using BD Cytofix/Cytoperm™ (BD Biosciences) before incubation with antibodies for intracellular staining. Final cell suspension was washed and acquired using a BD LSRFortessa™ cell analyzer (BD Biosciences). Manuel gating was carried out using Flowjo. Living CD45+ tumor-infiltrating immune cells were further analyzed to define specific immune cell subsets and their activation and differentiation status.

Histological Analysis

Briefly, tumor tissue was fixed in 4% paraformaldehyde overnight and embedded in paraffin. Sections (4 µm) were cut using a microtome (Leica) and mounted on glass slides. Samples were de-paraffinized and heat antigen retrieval was performed prior to immunostaining with antibodies specific for human CEA (Roche in house), human CD3 (Abcam, Cat No.: ab5690), and human PD-L1 (Ventana, Cat No.: 790-4905). Sections were counterstained with hematoxylin (Sigma Aldrich) and slides were scanned using Olympus VS120-L100 Virtual Slide Microscope. Quantification of percentage of positive stained tumor area for PD-L1 or CEA was performed in whole scans with Definiens software. Raw data was transferred to GraphPad software for analysis of significance. A total of five mice per treatment group was evaluated.

Cytokine/Chemokine Analyses

Cytokine/chemokine analyses from tumors of humanized mice were conducted using the Bio-Plex Pro™ Human Chemokine Panel, 40-Plex (Bio-Rad Laboratories AG, Cressier, Switzerland, Cat No.: 171AK99MR2). Small tumor fragments were snap frozen and whole protein was isolated in the presence of EASYpack Protease Inhibitor Cocktail (Roche; ref 5892970001) using the Precellys®24 Homogenizer and Bio-Plex® Cell Lysis Buffer following manufacturer instructions. Whole protein content was measured with BCA™ Protein Assay Kit (Thermo Scientific) before cytokine measurement was performed.

RNA Sequencing Analysis

High molecular weight RNA (>200 base pairs) was extracted and RNAseq libraries were generated and sequenced using the TruSeq® Stranded mRNA kit (Illumina®) as per manufacturer's instructions at Expression Analysis Inc.

Reads for each sample were processed using the following steps: First, reads were aligned to the human and mouse transcriptome (based on Ensembl v60) using Bowtie2 (42) with sensitive settings. In a second step, yet unmapped reads were aligned to the Human and mouse genome (hg19), and both mappings to genome and transcriptome were combined using in-house software. Reads mapped to both transcriptomes at the

same time were discarded from further analysis. Raw counts were used to create an R DGEList object [edgeR version 3.24.3 (43)].

Normalization factors were calculated using the calcNormFactors function. Genes were normalized by Trimmed Mean of M-values (TMM), and were subjected to DE analyses using the voom and lmFit functions in the limma package [version 3.38.3; (44)]. Gene set enrichment analysis was conducted using the fgsea R package [Version 1.8.0; (45)] with minSize=55, maxSize=500 an nperm=100000. Genes were ranked by the corresponding log2 fold-change and GO gene sets (C5) from MsigDB signaling database (46) were used.

Upregulation of PD-1/PD-L1 *In Vitro*

Surface expression of PD-1 on CD4+ or CD8+ T-cells and PD-L1 on surviving tumor cells was assessed after a classical tumor cell lysis assay. Briefly, peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated with standard techniques. MKN-45 target T-cells were plated at a density of 1.4×10^6 cells/well in flat-bottom 24-well plates 1 day before the assay. CEA-TCB or untargeted TCB were then added at concentrations ranging from 6.4 pM to 100 nM and PBMCs were added to obtain a final Effector : Target (E:T) ratio of 10:1 in a final volume of 1.1 ml per well. All experiments were performed in triplicate.

Target T-cell killing was assessed after 24 and 48 h of incubation by quantification of released lactate dehydrogenase (LDH) using an LDH detection kit (Roche Applied Science, Cat No.:11 644 793 001) according to manufacturers' instructions. Plates were read on a Spectramax ELISA reader and EC50 values were calculated.

Surviving tumor cells were detached Cell Dissociation Buffer (Gibco) and transferred into fresh 96-round-bottom well plates with the remaining PBMCs. FACS analyses were conducted on a BD Biosciences Fortessa system using fluorescently labeled antibodies specific for CD4 (BioLegend, Cat No.: 300532; BD Biosciences, Cat No.: 552838), CD8 (BioLegend, Cat No.: 301014; BD Biosciences, Cat No.: 563256), PD-1 (BioLegend, Cat No.: 329920), PD-L1 (BioLegend, Cat No.: 329708), and EpCAM (Miltenyi Biotech, Cat No.: 130-091-253).

To determine the impact of IFNγ on the PD-L1 expression on tumor cell lines, tumor cell lines were incubated for 48 h with 100 ng/ml IFNγ, and PD-L1 expression levels were determined by flow cytometry. Briefly, adherent cells were harvested using trypsin-EDTA (Life Technologies), washed with cell culture medium once and re-suspended with the respective cell culture medium with 100 ng/ml human IFNγ (PeproTech, 300-02). As reference, cells were plated in medium without IFNγ. After 48 h of incubation at 37°C, 5% CO2 in a humidified incubator, cells were harvested using cell dissociation buffer, washed with FACS buffer (PBS, 0.1% BSA) and stained using 40 µl FACS buffer containing 5 µl anti-PD-L1 antibody (BioLegend 329706) or 10 µl of the isotype control (mouse IgG2b, BD 556437). After 30 min at 4°C, cells were washed twice with FACS buffer and re-suspended in 200 µl FACS buffer containing 2% PFA to fix the cells for 20 min at RT in the dark. Finally, cells were analyzed using a BD FACS Fortessa, equipped with FACS Diva software.

In some experiments, anti- IFNγ blocking antibodies were added to the co-cultures to assess the impact on PD-L1 expression. Briefly, PBMCs from a healthy donor (obtained from Lonza) were co-cultured with MKN-45 or LS174T target cells. 25,000 target cells and 250,000 PBMCs were plated per well in flat-bottom 96-well plates, alternatively 25,000 target cells were plated without PBMCs. The anti-IFNγ antibody (InVivoMAb, Cat No.: BE0235) was added and cells were incubated for 30 min in a humidified incubator at 37°C and 5% CO_2. After 30 min the CEA-TCB, an untargeted TCB or media were added. The final concentration of the anti-IFNγ antibody was 5 ug/ml and that of the TCBs ranged from 100 nM to 6.4 pM in a final volume of 200 µl.

After 24, 48, or 72 h the PBMCs and tumor cells (adherent cells were detached using Cell Dissociation Buffer from Gibco) were transferred into fresh 96-well round-bottom plates. FACS analysis was conducted on a BD Biosciences Fortessa system using fluorescently labeled antibodies specific for CD4 (BioLegend Cat No.: 300532), CD8 (Biolegend Cat No.: 344704), CD25 (Biolegend Cat No.: 302614), CD69 (BioLegend Cat No.: 310934), PD-L1 (Biolegend Cat No.: 329706) and EpCAM (Miltenyi Biotec 130-091-254). Viable and dead cells were discriminated using Zombie Aqua Fixable Viability Kit (BioLegend Cat No.: 423102).

Statistical Considerations

Statistics are described in the legends.

Tumor growth inhibition values were calculated according to the equation:

$$TGI: \frac{100 - Av\left(T\_treatment^{[day\ x]} - T\_treatment^{[baseline]}\right)}{Av\left(T\_vehicle^{[day\ x]} - T\_vehicle^{[baseline]}\right)} \times 100$$

TCB-Treatment Score

Based on the Principal Component Analysis (PCA) of the ImmunoPD data, markers of PC1 (MIP-a, CXCL10, CXL13) and PC2 (CD8+ T-cells and PD-1+on CD8+ T-cells) were taken into account. The TCB treatment score summarizes the relative expression levels of MIPa, CXCL10 and CXCL13 as well as the levels of intra-tumor CD8+ T-cells and PD-1 expressing CD8+ T-cells. For a robust marker development, the estimation was based on the quintiles of the corresponding distributions of the markers in the present cohort. For every sample, depending on the relative expression of the particular marker, the marker got a discrete point ranging from 0 to 3: 0 for relative expression from the first quartile, up to 3 for the values from the last quartile of the corresponding distribution of marker values in the cohort. By applying this procedure, for each sample, an inhibitory receptor score in the range of $0 \leq$ inhibitory receptor ≤ 15 by summing up the points for the five corresponding markers was obtained. Finally, each inhibitory receptor score was normalized by 15. To compare the TCB-treatment score, the Wilcox test using JMP12 (JMP, Version 12; SAS Institute Inc., 1989–2007) have been applied.

RESULTS

Tumor Targeting of CEA-TCB

Dual-isotope SPECT/CT imaging of hematopoietic stem cell humanized NSG mice (HSC-NSG) bearing a human gastric adenocarcinoma xenograft tumor (MKN-45, displaying high CEA expression) showed tumor targeting and uptake of ^{111}In-CEA-TCB apparent at 4 h post-antibody injection, which continued to increase up to 120 h post single antibody injection (**Supplementary Figure 1A**). At the same time, the signal of ^{111}In-CEA-TCB in the blood stream and other organs gradually decreased. There was negligible tumor uptake of the ^{177}Lu-Untargeted-TCB injected simultaneously in the same mice. Quantitative analyses of the images revealed at least five-fold greater tumor uptake of ^{111}In-CEA-TCB than ^{177}Lu-Untargeted-TCB at all time-points. The amount of ^{111}In-CEA-TCB and ^{177}Lu-Untargeted-TCB in the spleen was identical, whereas the amount of ^{177}Lu-Untargeted-TCB in the blood pool was greater than that of ^{111}In-CEA-TCB (**Supplementary Figure 1B**). Together, imaging data indicated a tumor-specific targeting and accumulation of CEA-TCB over time along with clearance from the blood stream and other organs not expressing CEA.

CEA-TCB Treatment Reduces Tumor Growth and Generates a Highly Inflamed Tumor Microenvironment

Treatment of hematopoietic stem cell humanized NOG mice (HSC-NOG) bearing human MKN-45 tumor cells with CEA-TCB 2.5 mg/kg twice weekly reduced tumor growth by 62% compared with vehicle treated controls (**Figure 1A**). Flow cytometry analysis of tumors harvested after seven consecutive treatments showed that CEA-TCB treatment induced >10-fold increase in intra-tumor T-cells and >3-fold increase in the intra-tumor CD8/CD4 T-cell ratio (**Figures 1B, C, G**). Tumor-infiltrating T-cells demonstrated an activated phenotype as detected by increased expression of 4-1BB (CD137), an activation-induced T-cell costimulatory molecule (47), and PD-1, a hallmark of T-cell activation in this context (**Figure 1D**). Tumor-infiltrating T-cells also displayed a cytotoxic potential, exemplified by increased frequency of granzyme B (GZMB)-expressing cells, and proliferation, as evidenced by increased frequency of the Ki67 positive cells (**Figure 1D**). Treatment with CEA-TCB triggered secretion of pro-inflammatory cytokines (IFNγ, TNFα, IL-2) and several chemotactic molecules (CXCL9, CXCL10, CXCL11, CXCL13) indicating the generation of a highly inflamed tumor microenvironment (**Figure 1F**). CEA-TCB treatment also triggered upregulation of PD-L1 expression in tumors (**Figures 1G, H**). There were no changes related to T-cell activation or counts in the peripheral blood upon CEA-TCB treatment, further indicating that CEA-TCB activity is restricted to areas of CEA expression, such as in tumors (**Figure 1E**). The activity of CEA-TCB was additionally assessed in a CEA-expressing pancreatic tumor models (HPAF-II) in humanized NSG mice resulting in 72% of tumor growth inhibition, and confirmed the previous observations related to TCB mode of action consisting of strong increase of intra-tumor T-cells displaying an activated phenotype and increase of CD8/CD4 T-cell ratio (**Supplementary Figure 2**). The activity of CEA-TCB was further assessed in a genetically modified CEA424-SV40 TAg transgenic model, crossed with human CEACAM5 transgenic mice that spontaneously develop gastric tumors in the pyloric region (Steinhoff N et al., in preparation). CEA-TCB treatment led to a statistically significant reduction of CEA positive tumor area accompanied by a trend towards the increase of intra-tumor T cell infiltration and improvement survival (**Supplemental Figures 3A–D**).

The heatmap generated by combining cell surface and secreted T-cell activation markers (generated by flow cytometry and multiplex analysis from the MKN-45 experiment; **Figures 1A–G**) confirmed the clear separation of the CEA-TCB-treated animals and controls. CEA-TCB-treated tumors displayed a clear upregulation of several pro-inflammatory cytokines and chemokines [MIPα (CCL3), CXCL10, CXCL13, CXCL9, IL-16, and I-TAC (CXCL11)], an increase in intra-tumor CD3+, CD4+, CD8+ T-cells that express high levels of 4-1BB, PD-1, and upregulation of GZMB (**Figure 2A**). The relative Principal Component Analysis (PCA) confirmed distinct clustering of CEA-TCB-treated mice as compared to controls (**Figure 2B**), and further revealed the presence of two sub-clusters within the CEA-TCB-treated mice: one associated with high infiltration of activated T-cells (expressing high levels of PD-1, 4-1BB, and GZMB) and high expression of pro-inflammatory chemokines and cytokines (particularly CXCL10, CXCL13, and MIPα); the other associated with high infiltration of activated T-cells (expressing high levels of PD-1, 4-1BB, and GZMB) but low expression of pro-inflammatory chemokines and cytokines (CXCL10, CXCL13, and MIPα; **Figure 2A**). The correlation of these two inflamed tumor phenotypes with tumor volume or TCB activity with regards to tumor regression did not reveal a meaningful association (data not shown).

We further defined a *CEA-TCB-treatment score* (methods) with the aim to identify the parameters that most robustly characterize the CEA-TCB activity. The TCB-treatment score was generated based on the PCA of the ImmunoPD data considering top markers of Principal Component 1 (PC1) (MIP-a, CXCL10, CXCL13) and Principal Component 2 (PC2) (CD8+ T-cells and PD-1+ on CD8+ T-cells). CEA-TCB-treated tumors have a significantly higher CEA-TCB- treatment score ($p=0.0044$) compared to vehicle treated tumors (**Supplementary Figure 4**).

Identification of Gene Signature Associated With CEA-TCB Treatment

To more broadly characterize the molecular parameters associated with CEA-TCB treatment we performed bulk RNA sequencing of CEA-TCB-treated (seven consecutive treatments) and untreated tumors derived from MKN-45 tumor-bearing humanized mice (experiment from **Figure 1**; GEO accession number GSE155887). Similar to the ImmunoPD analysis described above, RNA sequencing analysis revealed a clear distinction between CEA-TCB-treated and control animals,

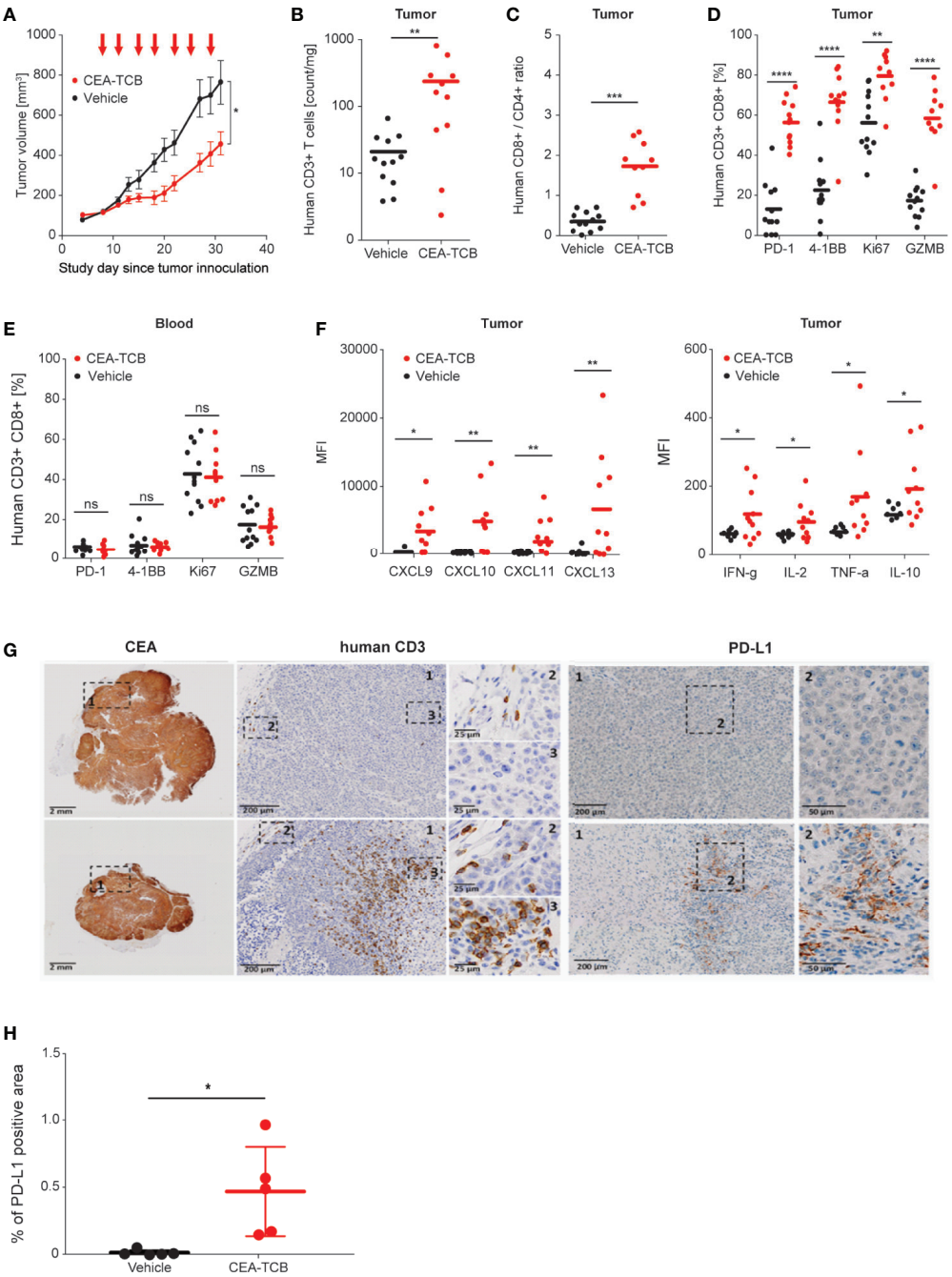


FIGURE 1 | Treatment with CEA-TCB induces tumor growth inhibition and leads to increased frequency of tumor-infiltrating human T-cells and a tumor-specific T-cell activation in MKN-45-bearing hematopoietic stem cell humanized mice. Hematopoietic stem cell humanized NOG mice were inoculated subcutaneously with 1 × 10^6 MKN-45 cells and treated with either buffer (vehicle; n=12) or with 2.5 mg/kg i.v. of CEA-TCB (n=12) twice weekly starting with a tumor volume of ~150 mm^3 (Day 8). At termination (Day 32), blood and tumors were harvested for subsequent flow cytometry, histological and cytokine analysis (ImmunoPD data). **(A)** Tumor growth kinetics revealed a tumor growth inhibition (TGI) of 62%. Arrows indicate treatments (seven in total). **(B–E)** Flow cytometry analysis of tumor and blood in vehicle- and CEA-TCB-treated animals showing the frequency of tumor-infiltrating T-cells **(B)** and ratio of CD8+ to CD4+ T-cells in the tumor tissue **(C)**, the expression of activation markers in tumor **(D)** and blood **(E)**. **(F)** Cytokine/chemokine expression in tumor lysates. **(G)** Representative histological staining for human CEA, CD3, and PD-L1 on paraformaldehyde fixed tumor samples from vehicle (upper row) and CEA-TCB-treated animals (lower row). **(H)** Quantification of PD-L1 staining by IHC. **(A)** Data are mean ± SEM; **(B–F, H)** solid bars represent mean values; p-values are two-tailed unpaired t-test; *p < 0.05; **p < 0.01; ***p < 0.001; ****p<0.0001.

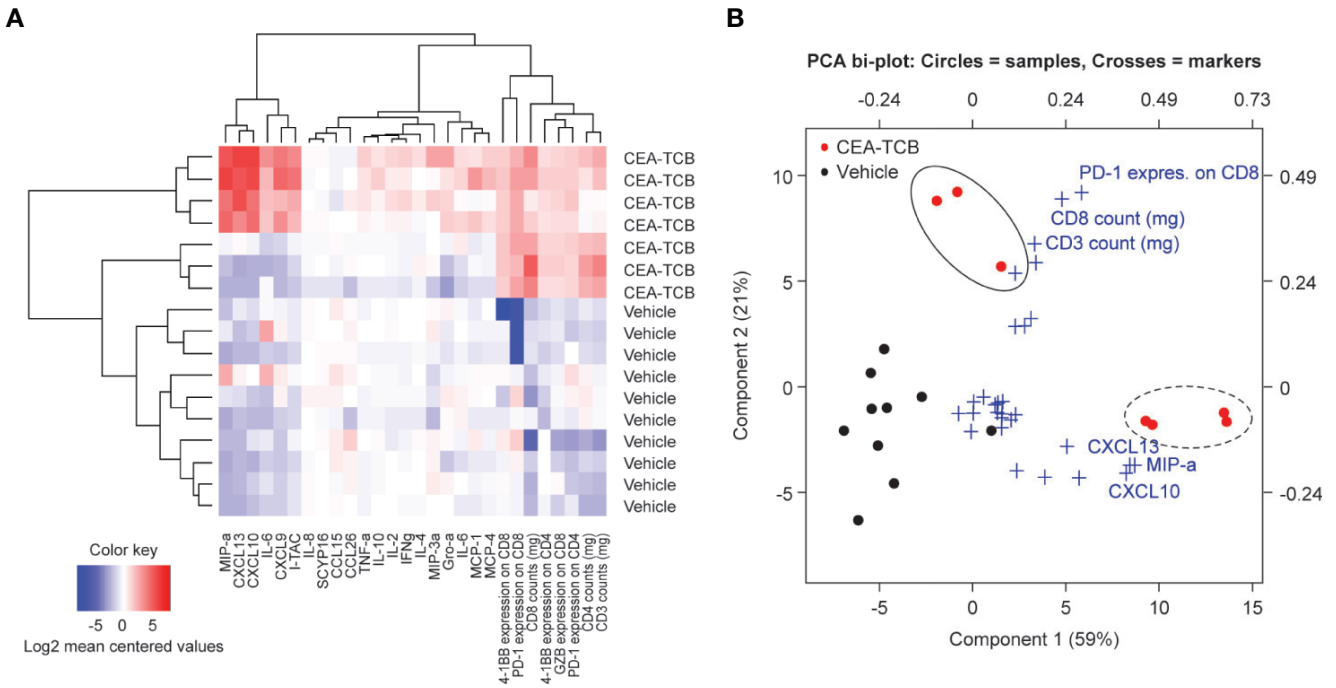


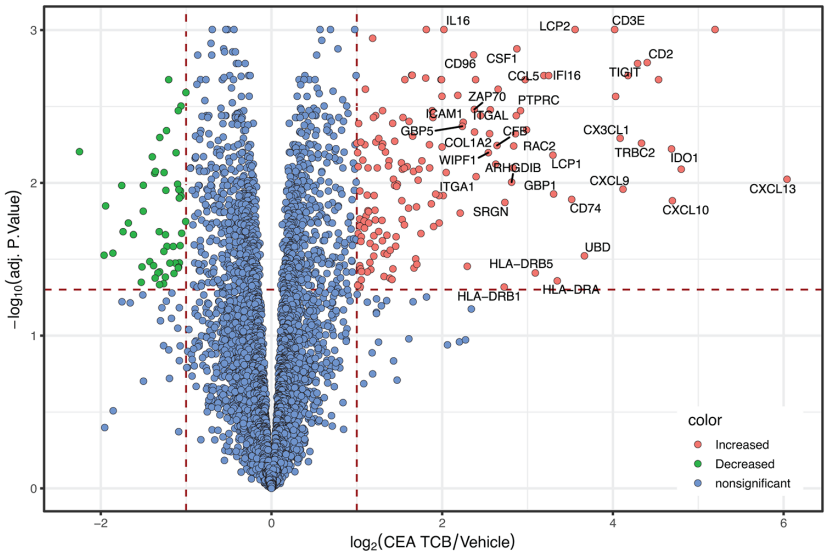
FIGURE 2 | ImmunoPD data defines CEA-TCB treatment cluster. **(A)** Heatmap of ImmunoPD data from the experiment in **Figure 1**. **(B)** Principal component analysis (PCA) of the ImmunoPD data reveals a distinct cluster for the CEA-TCB-treated mice. The CEA-TCB samples are defined by two groups: One group (dashed circle) is represented by samples having a high T-cell infiltration and a high expression of chemokines (*CXCL10*, *CXCL13*, and *MIPα*) and a second group (solid circles) is represented by samples having also a high T-cell infiltration but high exhaustion state and less expression of chemokines. Crosses showing the ImmunoPD marker and its impact on the Component 1 and 2.

with several genes being upregulated in tumors treated with CEA-TCB as compared to controls (**Figure 3**). The list of all genes that were found to have a significantly different expression (absolute log2 fold-change > 1 and an adjusted p-value < 0.05) between CEA-TCB-treated tumors and controls is provided in **Supplementary Table 1**.

Among the top 15 upregulated genes in CEA-TCB-treated tumors there were many reflective of a strong T-cell activation, migration, immune cell response (*CXCL13, GNLY, GZMB, CXCL10, IDO1, SLA, CD2, TRBC2, TIGIT, IL2RB, CXCL9, CX3CL1, CCL4L2*, and *CD3E*). Among the top downregulated ones we found *keratin 6A* and *keratin 20* (*KRT6A* and *KRT20*) suggestive of the reduction of tumor cells as the result of TCB-mediated killing (**Figure 3** and **Supplementary Table 1**). The Gene Set Enrichment Analysis (GSEA) further enabled identification of the main biological pathways upon CEA-TCB treatment and confirmed that the main Gene Ontology families that characterize the TCB response consist of T-cell activation (*Response to interferon gamma; Adaptive Immune Response; T-cell activation; Inflammatory Response; Activation of Immune response; Cytokine secretion*) and migration (*Leukocyte Migration; Regulation of Cell Adhesion*) (**Figure 3** and **Supplementary Figure 5**). Interestingly, we also noticed upregulation of many major histocompatibility class II molecules that are known to be expressed on antigen presenting cells (*HLA-DRA, HLA-DRB5*, and *HLA-DRB1*) along with *CX3CL1* (fractalkine, a known monocyte/T-cell attractant molecule) and *CSF1* (colony stimulating factor 1, macrophage) suggestive of myeloid cell recruitment and activation at tumor sites post TCB treatment. We also observed the upregulation of PD-1 and PD-L1 transcripts following CEA-TCB treatment as compared to controls (**Supplementary Figure 6**).

CEA-TCB Treatment Induces Upregulation of PD-1 and PD-L1 Expression; Combination of CEA-TCB With Anti-PD-L1 Blocking Antibody Enhances Its Efficacy in Stem Cell Humanized and Fully Immunocompetent Mice

Data shown in **Figures 1D, G, H** and **Supplementary Figure 6** provided evidence of PD-1 and PD-L1 upregulation on T-cells and tumors upon *in vivo* treatment with CEA-TCB. Additional evidence of the dose-dependent PD-1 upregulation on CD4 and CD8 T-cells as well as PD-L1 upregulation on tumor cells and T-cells upon CEA-TCB treatment was obtained from *in vitro* experiments (**Figures 4A–F**; **Supplementary Figure 6**). Incubation of the CEA-expressing MKN-45 target cells with human PBMC in presence of increasing concentrations of CEA-TCB led to the expected tumor cell lysis (**Figure 4A**). Flow cytometry analysis of co-cultured cells upon treatment revealed



goid	Pathway	adj. P-value	List of most regulated genes (up and down)
GO:0034341	Response to interferon gamma	0.0012	*CCL5, GBP4, OAS2, ICAM1, GBP5, CX3CL1, CIITA, TRIM22, GBP1, KYNU, IFITM2, UBD, IRF1, HLA-DRB5, HLA-DRA, HLA-DRB1*
GO:0002250	Adaptive immune response	0.0012	*CD3E, SLAMF6, C3, PTPRC, ZAP70, ICAM1, RFTN1, C1S, TRBC2, HAVCR2, CFI, CXCL13, CD74, PRF1, IRF1*
GO:0042110	T cell activation	0.0012	*CD3E, CCL5, CD2, TIGIT, SLAMF6, PTPRC, ZAP70, ICAM1, ITGAL, RAC2, LCP1, IDO1, HAVCR2, TNFRSF18, CD74, LAG3, TNFSF11, CORO1A, IRF1*
GO:0050900	Leukocyte migration	0.0012	*IL16, CSF1, CCL5, CD2, ZAP70, ICAM1, ITGAL, COL1A2, MMP1, CX3CL1, RAC2, PLA2G1B, CXCL13, TNFRSF18, CXCL9, CXCL2, ITGA1, CXCL10, CD74, ITGB2, CXCR4, TNFSF11, CORO1A, THBS1, ITGAM*
GO:0006954	Inflammatory response	0.0012	*CXCR6, CSF1, CD96, CCL5, IFI16, C3, PTPRC, ICAM1, ITGAL, GBP5, C2CD4B, CX3CL1, PTGS1, IDO1, HAVCR2, CIITA, CXCL13, CXCL9, CXCL2, CXCL10, HYAL1, ITGB2, CXCR4, TNFSF11, SERPINA1, SUCNR1, THBS1, ITGAM*
GO:0002253	Activation of immune response	0.0012	*LCP2, CD3E, IFI16, C3, PTPRC, ZAP70, RFTN1, C1S, TRBC2, CFB, WIPF1, HAVCR2, CFI, GBP1, ITGB2, WIPF3, PSMB9, ITGAM, SFTPA2, IRF1, HLA-DRB5, HLA-DRA, HLA-DRB1*
GO:0001816	Cytokine production	0.0012	*LCP2, CD3E, CD96, CD2, TIGIT, IFI16, SLAMF6, C3, PTPRC, GBP5, RFTN1, CX3CL1, CCBE1, IDO1, HAVCR2, PLA2G1B, GBP1, SRGN, CD74, LAG3, SUCNR1, THBS1, LTB, IRF1, UBE2L6*
GO:0031347	Regulation of defense response	0.0012	*CD96, CCL5, IFI16, SLAMF6, C3, PTPRC, GBP5, C2CD4B, RFTN1, CX3CL1, IDO1, HAVCR2, APOBEC3G, LAG3, ITGB2, TNFSF11, SUCNR1, PSMB9, ITGAM, SFTPA2, IRF1*
GO:0030155	Regulation of cell adhesion	0.0012	*CD3E, CSF1, CCL5, TIGIT, CYTIP, PTPRC, ZAP70, ICAM1, C2CD4B, CX3CL1, RAC2, IDO1, HAVCR2, ARHGDIB, CXCL13, TNFRSF18, GBP1, SPOCK2, CD74, LAG3, HYAL1, CXCR4, TNFSF11, CORO1A, THBS1, IRF1*
GO:0002274	Myeloid leukocyte activation	0.0012	*LCP2, CSF1, CCL5, CD2, C3, PTPRC, ITGAL, RAB31, CX3CL1, RAC2, HAVCR2, CD74, ITGB2, SERPINA1, SUCNR1, THBS1, ITGAM, UBD, PIGR, BATF2*

FIGURE 3 | RNA sequencing data showing differentially expressed genes and Gene Ontology pathways between CEA-TCB-treated mice and controls. Tumors from the experiments in Figure 1 were harvested after seven consecutive treatments and subjected to RNA sequencing. The Volcano-plot (upper panel) displays the log2 gene expression fold change between CEA-TCB vs vehicle group (X axis) in function of the –log10 adjusted p-value using Benjamini & Hochberg correction (Y axis). Gene names are shown for genes having a log2 fold-change >2 and an adjusted p-value < 0.05. The Gene Ontology families generated considering the most deregulated genes upon CEA-TCB treatment (adj.pval < 0.05) are summarized in the table below.

dose-dependent upregulation of PD-L1 on tumor cells (**Figure 4B**) and dose-dependent upregulation of PD-1 (**Figures 4C, D** and **Supplementary Figures 7A, B**) and PD-L1 (**Figures 4E, F** and **Supplementary Figures 7C, D**) on CD4+ and CD8+ T-cells compared with cells incubated with untargeted TCB control. IFNγ is the main mediator of PD-L1 upregulation on tumor cells (**Supplementary Figure 8A**) and is released by CEA-TCB activated T cells in co-culture with tumor cells (**Supplementary Figures 8B, C**). Treatment of tumor cells with CEA-TCB in the absence of immune cells did not lead to PD-L1 upregulation on tumor cells, further confirming the key role of activated immune cells in secreting IFNγ (**Supplementary Figures 8B, C**). Further experiments corroborated the key role of IFNγ demonstrating that blocking of IFNγ by means of neutralizing antibodies reduced the PD-L1 upregulation on tumor cells resulting from CEA-TCB-mediated T cell activation (**Supplementary Figures 8D, E**).

The upregulation of PD-1 on T-cells and PD-L1 on tumor and T-cells following *in vitro* and *in vivo* TCB treatment led us to investigate whether combining CEA-TCB with PD-L1 blocking antibody could enhance the anti-tumor efficacy of CEA-TCB. We initially investigated the activity of this combination in HSC NOG mice bearing MKN-45 tumors. Treatment with CEA-TCB and anti-PD-L1 blocking antibody improved anti-tumor activity compared with either agent alone; in addition, the onset of tumor regrowth was significantly delayed in the combination group

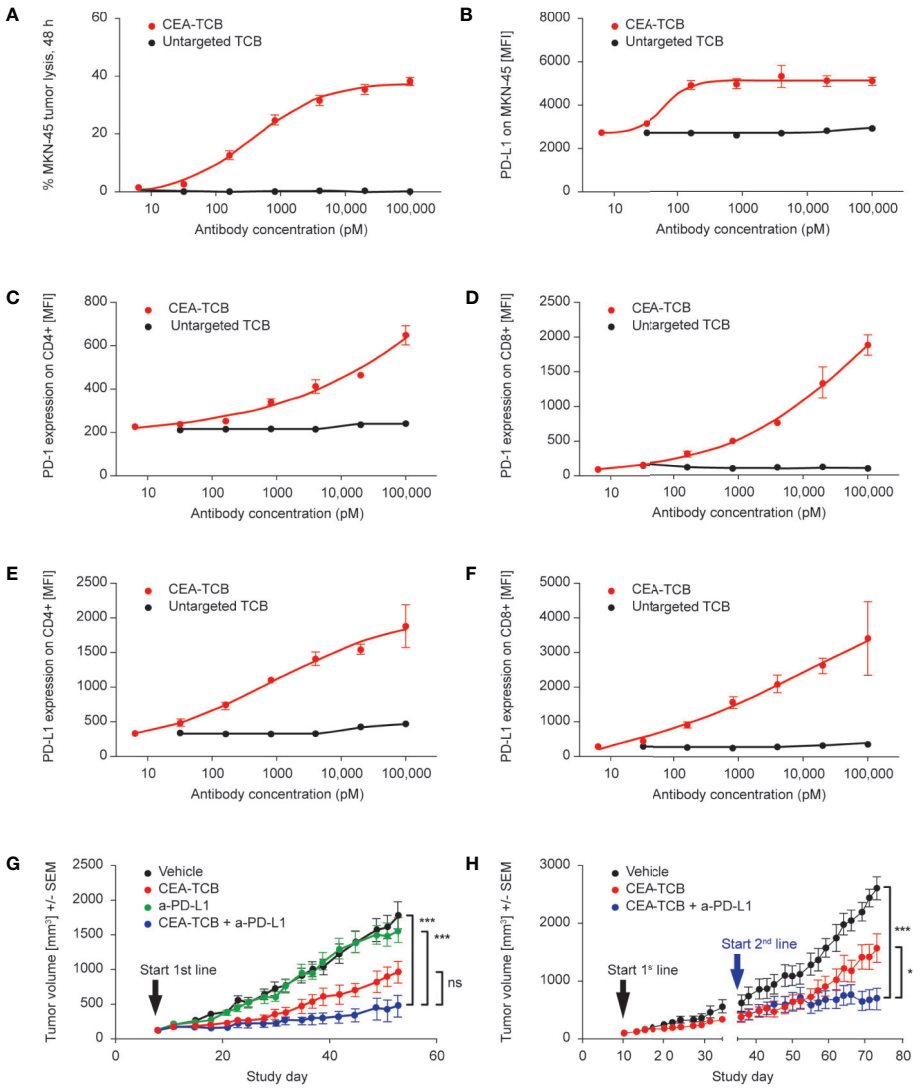


FIGURE 4 | TCB-mediated cytotoxicity induces the expression of PD-1 and PD-L1; blockade of PD-1/PD-L1 axis improves the efficacy of CEA-TCB in humanized mice. **(A–F)** Example of CEA-TCB-mediated tumor cell lysis *in vitro* leading to T-cell activation with parallel upregulation of PD-1 (on both CD4+ and CD8+ T-cells) and PD-L1 (on MKN-45 tumor cells). Data are the mean and standard deviation of triplicate experiments. **(A)** Tumor cell lysis as measured by LDH release assay in a co-culture assay of human PBMC, MKN-45 tumor cells [effector:target (E:T) ratio: 10:1] in presence of increasing concentrations of either CEA-TCB or an untargeted TCB after 48 h of incubation. **(B)** Flow cytometry analysis for PD-L1 expression (MFI) on MKN-45 cells recovered after TCB-mediated killing from co-culture assays. **(C, D)** Flow cytometry analysis for PD-1 expression (MFI) on human CD4+ and CD8+ T-cells recovered after TCB-mediated killing from co-culture assays. **(E, F)** Flow cytometry analysis for PD-L1 expression (MFI) on human CD4+ and CD8+ T-cells recovered after TCB-mediated killing from co-culture assays. **(G, H)** Hematopoietic stem cell humanized NOG mice were inoculated subcutaneously with 1×10^6 MKN-45 cells and treated with i.v. buffer (vehicle) twice weekly or with 2.5 mg/kg i.v. CEA-TCB twice weekly or 10 mg/kg i.v. of anti-PD-L1 once weekly, or with a combination of CEA-TCB plus anti-PD-L1 (given at the same dose and schedule as in monotherapy groups) starting with a tumor volume of ~150 mm^3. Tumor growth kinetics are shown as mean ± SEM for all treatment groups (n=9 mice per group). **(G)** Combination treatment of CEA-TCB and anti-PD-L1 started from the beginning (Day 8; 1st line treatment). **(H)** Combination treatment of CEA-TCB with anti-PD-L1 started once animals progressed to CEA-TCB monotherapy treatment (on Day 35; 2nd line treatment). p-values are one-way ANOVA with Tukey's multiple comparison correction: ns, not significant; $^{**}p < 0.01$, $^{***}p < 0.001$.

compared with anti-PD-L1 single-agent treatment (**Figure 4G**). Although the difference of the combination treatment compared with CEA-TCB monotherapy was only close to being significant, data support a trend for improved efficacy of the combination effect. Stronger anti-tumor activity was achieved when the combination started from the beginning (e.g. from the first treatment cycle) (**Figure 4G**) compared with combination that started after progression to CEA-TCB monotherapy treatment (**Figure 4H**).

The efficacy of the combination of CEA-TCB plus a PD-L1 blocking antibody was further assessed in fully immunocompetent model consisting of human CEA transgenic (huCEA Tg) C57BL/6J

mice bearing a syngeneic colorectal tumor line (MC38) stably expressing human CEA (MC38-hCEA). Treatment with murine surrogate of CEA-TCB (muCEA-TCB) and of anti-PD-L1 blocking antibody (muPD-L1) led to more rapid, more pronounced and sustained tumor growth inhibition as compared to the respective monotherapy treatment groups (**Figure 5A**).

Flow cytometry analysis of treated tumors, harvested 24 h after third infusion of the molecules, revealed that, similar to studies in humanized mice, CEA-TCB monotherapy increased the frequency of intra-tumor T-cells with preferential increase of CD8 T-cells over CD4 T-cells, leading to an increased ratio of intra-tumor CD8/CD4 and CD8/Tregs (**Figure 5B**). The majority of CD8 T-cells displayed a cytotoxic effector and effector memory phenotype and a significant fraction of CD8 T-cells (35%) displayed triple expression of PD-1+Tim3+Lag3+ (putatively exhausted cells). In addition, CEA-TCB treatment increased the frequency of intra-tumor CD4 and CD8 T-cells expressing CXCR3, a key receptor regulating T-cell chemotaxis (**Figure 5B**). Interestingly, combination treatment of CEA-TCB and anti-PD-L1 blocking antibody increased the intra-tumor frequency of both CD4 and CD8 T-cells compared with monotherapies and vehicle control. This led to a similar frequency of cytotoxic effector and effector memory cells, but a lower frequency of triple positive, putatively exhausted PD-1+Tim3+Lag3+ CD8 T-cells (15% in combination vs 35% in CEA-TCB monotherapy) and higher frequency of intra-tumor CXCR3+ CD8+ T-cells (11% in combination vs 5% in CEA-TCB monotherapy) (**Figure 5B**). Together, the intra-tumor T-cell phenotype upon CEA-TCB and anti-PD-L1 combination treatment is reflective of T-cells having higher propensity for recruitment and putatively lower exhaustion status, and may reflect a higher recruitment of fresh T-cells from the peripheral blood.

Combination With Anti-PD-L1 Also Enhances the Efficacy of CD20-TCB in Stem Cell Humanized Mice

We further demonstrated the value of combining a TCB antibody with PD-L1 blockade for hematological malignancies using CD20-TCB (glofitamab), another "2:1" format TCB targeting CD20 on B cells and CD3 on T-cells (9, 48). Glofitamab is currently under clinical development in patients with relapsed or refractory B-cell non-Hodgkin lymphoma. To

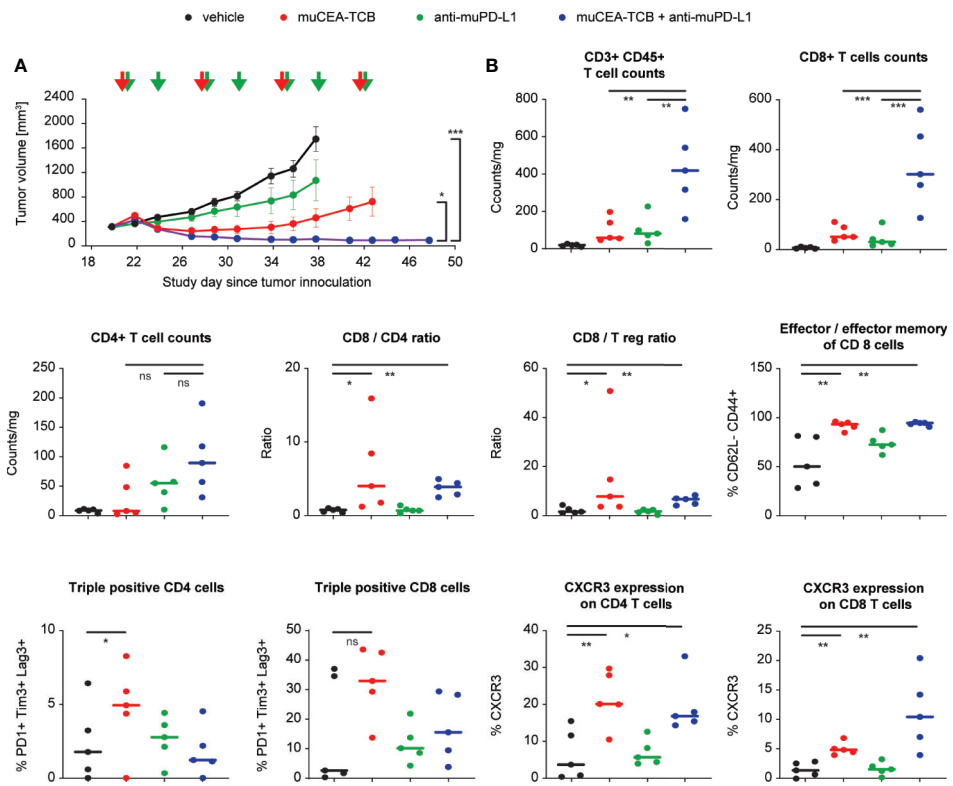


FIGURE 5 | Blockade of PD-1/PD-L1 axis improves the efficacy of CEA-TCB in immunocompetent mice. **(A)** Immunocompetent human CEA transgenic (huCEA Tg) C57BL/6J mice were inoculated subcutaneously with 0.5 × 10^6 MC-38-huCEA cells and treated with i.v. buffer (vehicle), murine surrogate of CEA-TCB (muCEA-TCB; 2.5 mg/kg i.v. once weekly), murine surrogate of anti-PD-L1 (10 mg/kg i.v. initial dose followed by 5 mg/kg i.p. twice weekly), or with a combination of muCEA-TCB and a-muPD-L1 (same dose and schedule as in monotherapy groups). Treatment started with a tumor volume of 200–400 mm^3 (Day 20). Arrows indicate treatments. Tumor growth kinetics are shown as mean ± SEM for all treatment groups (n=16 mice per group). Combination group vs muCEA-TCB: p=0.023 and vs vehicle: p<0.001; one-way ANOVA with Tukey's multiple comparison correction done after five treatments (Day 38). **(B)** 24 h after the third treatment (Day 29), scout mice were sacrificed, tumor tissue was obtained and analyzed for T-cell infiltration and phenotype. p-values are one-way ANOVA with Tukey's multiple comparison correction: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

better evaluate the effect of the combination, HSC NSG mice bearing an aggressive human DLBCL cell line (WSU-DLCL2) were treated with a suboptimal dose of CD20-TCB (0.15 mg/kg). This resulted in suboptimal anti-tumor activity compared to its optimal dose (9), as monotherapy or in combination with an anti-PD-L1 blocking antibody. While monotherapy treatment with CD20-TCB or anti-PD-L1 blocking antibody did not show anti-tumor efficacy, the combination treatment led to tumor growth inhibition (**Supplementary Figure 9**).

DISCUSSION

The current study was undertaken to expand our understanding of cellular and molecular features associated with TCB activity and to address one of the key adaptive resistance mechanisms related to TCB activity, namely PD-1/PD-L1 axis upregulation, similarly to what has been described for checkpoint inhibitors (30).

The efficacy and mode of action of single-agent CEA-TCB was evaluated in different preclinical CEA-expressing mouse tumor models. These comprised hematopoietic stem cell humanized NOG mice bearing human gastric and pancreatic tumors and immunocompetent human CEA transgenic C57BL/6J mice (hCEA Tg mice) bearing a murine colorectal cancer tumor line (MC38) or crossed with genetically modified CEA424-SV40 TAg transgenic mice that spontaneously develop gastric tumors in the pyloric region. The former represent a hyper-mutated and highly inflamed form of colorectal cancers (MSI^{hi} CRC) (49) transfected to stably express human CEA (MC38-hCEA), the latter an aggressive form of murine gastric cancer with immune desert phenotype, which is poorly responsive to cancer immunotherapy treatment (Steinhoff N et al., in preparation).

In all models, single-agent CEA-TCB slowed the growth of tumors compared with controls. Treatment of mice bearing CEA-positive tumors with CEA-TCB led to a 2 to >10-fold increase in tumor-infiltrating T-cells (depending on the tumor and mouse model). The tumor-infiltrating T-cells displayed a highly activated and proliferating phenotype, with tumors displaying a highly inflamed microenvironment as evidenced by increased levels of several pro-inflammatory cytokines and chemokines. Notably, anti-tumor efficacy along with tumor inflammation and increases of activated intra-tumoral T-cells was obtained in response to CEA-TCB treatment, even in settings with low pre-existing baseline tumor immune cell infiltration. This indicates that unlike other immunotherapies, CEA-TCB has the potential to be efficacious in patients with poor pre-existing inflammation. This is particularly relevant for patients with low frequency of pre-existing intra-tumoral CD8 + cells, who respond poorly to cancer immunotherapy (50) and particularly for the vast majority of human (CEA-expressing) colorectal cancer tumors with proficient mismatch repair (MMR) or with microsatellite stable (MSS) tumors who do not benefit from immunotherapy. Colorectal tumors with microsatellite instability (MSI) are typically more antigenic and have greater infiltration of CD8+ cells than MSI-negative tumors (51, 52).

A molecular signature of TCB treatment was identified consisting of pro-inflammatory cytokines/chemokines, and higher frequency and activation of T-cells. The signature appeared to be robust, as components of the signature were confirmed using complementary techniques: RNA expression analysis and protein expression as determined by flow cytometry and multiplex analysis. In particular, *CXCL9* and *CXCL10* were identified by both methods as the key molecules significantly upregulated by CEA-TCB treatment compared to controls. CXCL9 and CXCL10 are potent pro-inflammatory chemokines and chemoattractants for multiple immune effector cells, including NK cells, monocytes/macrophages and T-cells by binding to the CXCR3 receptor expressed on the same cells (53, 54). In line with this, CEA-TCB treatment also increased the frequency of intra-tumor CXCR3+ CD8+ and CD4+ T-cells, corroborating the relevance of the CXCL10-CXCR3 axis in mediating the attraction of T-cells leading to increase of intra-tumor T-cell infiltration upon TCB treatment (25) (and unpublished data). It will be interesting to investigate the prognostic value of the TCB-treatment score in biopsies obtained from the ongoing interventional trial of the combination of CEA-TCB (cibisatamab) and atezolizumab in previously treated metastatic colorectal adenocarcinoma patients (NCT03866239).

A clear upregulation of both PD-1 (on CD4 and CD8 T-cells) as well as PD-L1 (on tumor cells and CD4 and CD8 T-cells) was detected in response to CEA-TCB treatment, indicative of the PD-1/PD-L1 axis being one of the adaptive resistance mechanisms related to TCB activity (8, 9, 25–29). Combination of TCBs with anti-PD-L1 blocking antibody (in different tumor and mouse models and using different TCBs targeting both CEA (solid tumors) and CD20 (hematological malignancies) consistently translated into superior anti-tumor efficacy and stronger tumor growth inhibition when compared to either agent given as monotherapy. Better tumor growth inhibition was obtained when the two agents were combined simultaneously from the first treatment cycle, as compared to starting the combination when tumors progressed to CEA-TCB monotherapy. This finding is consistent with previous *in vitro* data with a CEA BiTE MEDI-565/AMG 211 that showed that T-cell killing was maximized when dual blockade of PD-1 and PD-L1 was applied early (26). Interestingly, the combination of CEA-TCB and an anti-PD-L1 blocking antibody led to increased frequency of intra-tumor CD4 and CD8 T-cells displaying a cytotoxic effector and effector memory phenotype; at the same time, the combination treatment lowered the frequency of putatively exhausted T-cells (characterized by co-expression of PD-1+Tim3+Lag3+ CD8 T-cells) and increased the frequency of T-cells having migratory capacity (characterized by CXCR3+ expression on CD8+ T-cells). Taken together, the intra-tumor T-cell phenotype upon CEA-TCB and anti-PD-L1 combination treatment is reflective of T-cells having higher propensity to migrate and putatively lower exhaustion status, and may indicate a stronger recruitment of fresh T-cells from the periphery.

These pre-clinical data support the rationale for the clinical investigation of CEA-TCB and atezolizumab, which is currently

in Phase Ib (NCT03866239). Preliminary results of clinical activity indicated promising anti-tumor efficacy in patients with CEA+ solid tumors (mostly colorectal cancer) when cibisatamab was combined with the anti-PD-L1 antibody atezolizumab (55). Comparison of pre-treatment and on-treatment patient tumor biopsies (most of which came from MSS CRC patients with a non T-cell inflamed immunophenotype prior to treatment) indicated that cibisatamab and atezolizumab combination treatment led to the increase of intra-tumor proliferating T-cells, increase of PD-1+ T-cells, upregulation of PD-L1 expression on immune cells, and reduction of CEA expressing tumor cells (56, 57), corroborating pre-clinical findings presented in the current study.

In conclusion, the data of the current study expand our knowledge of the cellular and molecular features associated with TCB activity, and provide evidence that the PD-1/PD-L1 axis is one of the adaptive resistance mechanisms associated with TCB activity. This adaptive resistance mechanism can be managed by the combination of TCB with anti-PD-L1 (or anti-PD-1) blocking antibodies translating into more efficacious anti-tumor activity and prolonged control of the tumor outgrowth. However, the data also show that tumors continue to progress despite the anti-PD-L1 combination treatment, suggesting that additional mechanisms, beyond the PD-1/PD-L1 axis, contribute to tumor escape. The elucidation of such mechanisms, most likely contributed to by both tumor cells and different immune cell subsets, by using high dimensional single cell approaches for tumor analysis, will constitute an important milestone in our understanding of additional resistance mechanisms to immunotherapy and novel combination approaches for efficient tackling of the same.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155887.

ETHICS STATEMENT

This study involving laboratory animals was reviewed and approved by the Institutional Animal Care and Use Committee of the Preclinical Pharmacology Department, Roche Innovation Center Zurich, Schlieren, Switzerland. This study was performed in accordance with the animal research protocols approved by the local government (Kantonale Verwaltung Veterinäramt kant. Zürich, Switzerland; license ZH193/2014). All animals were handled in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations, Gesellschaft für Versuchstierkunde/Society of Laboratory Animal Science (GV-Solas) and the Tierschutzgesetz (TierSchG).

AUTHOR CONTRIBUTIONS

TF, MBi, and LF were in involved in the design and generation of *in vitro* data. JS and SC designed, supervised, and interpreted the *in vivo* and *ex vivo* studies. MP designed and supervised *in vivo* studies in immunocompetent mice. EB, AS, MK, ML, and NS conducted all *in vivo* and *ex vivo* experiments. VN generated all histology results. TN designed and supervised the SPECT/CT imaging study. AR supervised and analyzed the bulk RNAseq data. MBa, CK, and PU supervised the project. MBa and JS wrote the manuscript. MBa contributed to experimental design and data interpretation of all *in vitro* and *in vivo* studies. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.575737/full#supplementary-material

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