A novel therapeutic bispecific format based on synthetic orthogonal heterodimers enables T cell activity against Acute Myeloid Leukemia

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Article

Keywords:

Posted Date: October 25th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2135768/v1

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Version of Record: A version of this preprint was published at Oncogene on November 10th, 2022. See the published version at https://doi.org/10.1038/s41388-022-02532-2.
Abstract

Many therapeutic bispecific T-cell engagers (BiTEs) are in clinical trials. A modular and efficient process to create BiTEs would accelerate their development and clinical applicability. In this study, we present the design, production, and functional activity of a novel bispecific format utilizing synthetic orthogonal heterodimers to form a multichain modular design. Further addition of an immunoglobulin hinge region allowed a stable covalent linkage between the heterodimers. As proof-of-concept, we utilized CD33 and CD3 binding scFvs to engage leukemia cells and T-cells respectively. We provide evidence that this novel bispecific T-cell engager (termed IgGlue-BiTE) could bind both CD3+ and CD33+ cells and facilitates robust T-cell mediated cytotoxicity on AML cells in vitro. In a mouse model of minimal residual disease, we showed that the novel IgGlue-BiTE greatly extended survival, and mice of this treatment group were free of leukemia in the bone marrow. These findings suggest that the IgGlue-BiTE allows for robust simultaneous engagement with both antigens of interest in a manner conducive to T cell cytotoxicity against AML. These results suggest a compelling modular system for bispecific antibodies, as the CD3- and CD33-binding domains can be readily swapped with domains binding to other cancer- or immune cell-specific antigens.

Introduction

Bispecific proteins can be designed to engage two different cell types (e.g., a T cell and a cancer cell, or a natural killer cell and a cancer cell), or to combine two different functionalities (e.g., binding a cancer cell and activating a T cell). Bispecific antibodies (BsAbs) are a class of non-natural molecules that are specifically designed to recognize two different epitopes on the same or different antigens that are present on the same or two different cells \(^2\). However, designing and producing a functional bispecific protein in a concentrated manner has been historically challenging \(^9,10\). Improved knowledge of immunoglobulin crystal structure and advanced genetic engineering tools have created a field of “antibody engineering” in which multiple formats are used for assembling novel bispecific formats resulting in over 100 different formats \(^2\). With the advent of technologies such as knobs-into-holes, strand-exchange engineered domain, and electrostatic steering, the desired BsAb combination can be produced at a yield of greater than 90\% \(^9,10\). Technologies such as CrossMab and Orthogonal Fab, which utilize a common heavy chain with enforcement of a desired light chain can also generate BsAbs \(^9,10\). Some of the current BsAb production technologies also further suffer from a “steric” challenge: if the two binding moieties are contained in the same continuous chain, then the binding of one moiety may hinder the binding or affinity of the second. Indeed, previous studies have shown that the placement of binding regions at the C-terminus or internal positions of a fusion protein reduces target affinity compared to those at the N-terminus \(^6,7,11\). Therefore, a more effective design would allow for placement of the target-binding and effector-binding moieties on the N-terminus of separate protein strands, thus mimicking the naturally occurring IgG antibody structure. Several formats that utilize self-associating peptides were described including helix, helix-1 turn helix-2, GCN4 leucine zipper, JUN leucine zipper, and FOS leucine zipper \(^4,8,16\). However, there is no one format that is considered best as each has its own inherent advantages and disadvantages relative to the intended application \(^2,9,10\). The size, arrangement, number of valency sites, orientation and type of binding molecule all contribute to the pharmacokinetics and pharmacodynamics of the BsAb \(^2\).

In this study, we designed a novel bispecific molecule utilizing an orthogonal heterodimer scaffold composed of a highly specific four-helix structure formed by two synthetic polypeptide sequences in order to present two scFvs at the N-terminus \(^3\). In this proof-of-concept study, utilizing a novel BsAb with scFvs specific to CD33 and CD3, we demonstrate the ability of such a molecule to bind both cell types, to facilitate specific T cell cytotoxicity in vitro and to extend survival in vivo in a mouse model of AML with minimal residual disease. We believe this represents a modular approach to creating bispecific molecules, wherein scFvs binding CD33 and CD3 may be substituted for binders to a myriad of antigens of both malignant and immune cells. This novel BsAb format would be particularly appealing in cases where steric hindrance makes the design of a single-chain BsAb challenging.

Materials And Methods

Amino Acid Sequences

\( \alpha \text{CD33-6DMPa-Hinge} \)
MYRMQLLSCIALSLALVTNSEIVLTQSPGSLAVSPGERVTMSCKSSQSVFFSSSQKNYLAWYQQIPGQSPRLIYASTRESGVPDRTGSGSGTDTFTLTISSVQPEDLAIYCHQYLSSRTFGQGTKLEIKRGTSTSGSGPGSSEGSTKGVQVQLQPGAEVVKPGAVKMSCKKASGYTFTSYYIHWIKOTPGQGLEWGVYIPGNDDISYNKQFQKATLTDKSSTTAYMQLLSLSEDVYYCCAREVRLRYFDVWGQGTTVTVSSSSAGGGGSGGGSGGGSGGTKEDELIERQIKIERAQEHRQEQILEEEIERIIRKPGSSEEAMKRMKLLLEELRLLLKELLESEASQLLYERQERKCCVECPPCPEQQLSEEDLHHHHHH


αCD3-6DMPb-Hinge

MYRMQLLSCIALSLALVTNSEIVLTQSPGSLAVSPGERVTMSCKSSQSVFFSSSQKNYLAWYQQIPGQSPRLIYASTRESGVPDRTGSGSGTDTFTLTISSVQPEDLAIYCHQYLSSRTFGQGTKLEIKRGTSTSGSGPGSSEGSTKGVQVQLQPGAEVVKPGAVKMSCKKASGYTFTSYYIHWIKOTPGQGLEWGVYIPGNDDISYNKQFQKATLTDKSSTTAYMQLLSLSEDVYYCCAREVRLRYFDVWGQGTTVTVSSSSAGGGGSGGGSGGGSGGTKEDELIERQIKIERAQEHRQEQILEEEIERIIRKPGSSEEAMKRMKLLLEELRLLLKELLESEASQLLYERQERKCCVECPPCPEQQLSEEDLHHHHHH


Buffers

The following buffers were used in various experiments described below. PBS – Dulbecco's Phosphate Buffered Saline (Gibco). Flow cytometry/FACS buffer – Dulbecco's PBS with 4% fetal bovine serum. PBST – PBS with 0.05% Tween 20 (Fisher Scientific). TBST – Tris buffered saline with 0.05% Tween 20 (Fisher Scientific).

Structural prediction with AlphaFold multimer

Structural prediction of monomer and heterodimer was performed in AlphaFold2-multimer. We used ColabFold, a Jupyter Notebook hosted by Google, that allows for prediction of protein structures and complexes by combining the fast homology search of MMseqs2. We used default setting. The prediction generated 5 models, and all were predicted as heterodimers. The structure shown in Fig. 1 is the rank 1 model. The model were visualized and images were captured using ChimerX.

Cell Culture

All cells were held at 37°C in a humidified environment with 5% CO2. Cells were tested for mycoplasma with a LookOut Mycoplasma PCR Detection Kit (Sigma Aldrich). CHO-K1 cells, purchased from ATCC (Manassas, Virginia) were maintained in F12K medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P-S). HL-60 and MOLM14 were maintained in RPMI 1640 Medium (Gibco, Waltham, Massachusetts) with 20% FBS and 1% P-S. CD3+ T-cells were selected from fresh or frozen human peripheral blood mononuclear cells (STEMCELL Technologies, Vancouver) using REAlease CD3 MicroBead Kit (Miltenyi Biotec, Auburn, California), following manufacturer protocols. Purified CD3+ cells were cultured in OpTimizerTM CTSTM T-Cell Expansion SFM medium (Gibco) supplemented with interleukin-7 (IL-7) 10ng/mL and interleukin-15 (IL-15) 5ng/mL, in G-Rex 6-well plates (Wilson Wolf, New Brighton, Minnesota). Purified T-cells were activated on the same day as purification using CD3/CD28 Dynabeads (ThermoFisher, Waltham, Massachusetts), following manufacturer protocols.

Transfection

CHO-K1 cells were transfected according to a modified version of the manufacturer protocol for Lipofectamine 3000 (ThermoFisher, Waltham, Massachusetts). 1.2 x 10^7 CHO-K1 cells were plated in 15-cm tissue culture-treated dish 24 hours prior to transfection. To prepare anti-CD33/anti-CD3 dimers, 40 µg of each pcDNA plasmid were mixed and diluted in 3 mL of Opti-MEM (Gibco). 160 µL of P3000TM reagent was added to complete the P3000TM-DNA mixture. Separately, two dilutions of Lipofectamine® 3000 were prepared, with either 50 µL or 100 µL of Lipofectamine® 3000 added to 1.5 mL of Opti-MEM. 1.5 mL of P3000TM-DNA mixture was added to each dilution of Lipofectamine® 3000-Opti-MEM, and allowed to incubate for 5 minutes. Following incubation, complete lipid-DNA complex was added dropwise to CHO-K1 cells, beginning with the lower Lipofectamine® 3000 dilution. CHO-K1 cells were returned to the incubator for 72–96 hours. Finally, cell culture supernatant was collected,
centrifuged to separate from any detached cells, and supplemented with phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM to inhibit serine protease activity during purification.

**Immunoprecipitation**

TALON Metal Affinity Resin (Takara Bio USA, San Jose, California) was added to a microcentrifuge tube, and diluted to 1mL with PBST. For each 15cm-plate, about 20mL of supernatant is collected, and 200 µL of TALON Metal Affinity Resin is prepared and washed with PBST. Resin was vortexed to mix, and centrifuged at 10,000 g for 5 minutes at 4°C. Supernatant was discarded, and resin was resuspended in 1mL of PBST. Resin was vortexed to mix, and centrifuged at 10,000g for 5 minutes at 4°C. After discarding supernatant, resin was added to cell culture supernatants prepared as described above. Samples rotated overnight at 4°C. Samples were centrifuged at 500g for 5 minutes at 4°C, and supernatant was aspirated. Resin was resuspended in PBST, rotated for 10 minutes at 4°C, and centrifuged as done previously. This washing process was repeated for a total of 3 washes. 300 µL of elution buffer, 150 mM imidazole in PBS, per 100 µL of resin was added to the resin bed, and samples rotated at least 1 hour at 4°C. Samples were centrifuged at 500 g for 5 minutes at 4°C, and gently resuspended by pipetting before transfer to Micro Bio-Spin chromatography columns (Bio-Rad Laboratories, Hercules, California). Columns were placed in microcentrifuge tubes, and centrifuged at 10,000 g for 1 minute at 4°C. Eluates from TALON resin were diluted to a final volume of 6 mL in PBS, and added to 30kDa MWCO protein concentrator (ThermoFisher). Samples were centrifuged at 3,000g for 15 minutes. Flow-through was discarded from the lower chamber of the concentrator, and concentrated samples were diluted in 5mL of PBS before centrifugation under the conditions described above. Concentrated samples containing His-purified experimental constructs were collected, and either used immediately in subsequent experiments or frozen until use at -20°C.

**Western Blot**

CHO-K1 cells were transfected in 15-cm tissue culture-treated plates as described above. Samples were mixed with 2X-Laemmli sample buffer (Bio-Rad) containing β-mercaptoethanol at a final concentration of 355 mM, and heated at 95°C for 5 minutes. Non-reducing samples were mixed in 2X-Laemmli sample buffer without β-mercaptoethanol. 25 µL of sample were transferred to the wells of a Novex 4–20% tris-glycine mini gel (Invitrogen, Waltham, Massachusetts) submerged in tris-glycine-SDS running buffer (Bio-Rad). A constant voltage of 225V was applied until the dye front migrated to the bottom of the gel. Gels were removed from the cassettes, and placed in tris-glycine transfer buffer (Bio-Rad) with 10% methanol. 0.2 µm pore PVDF membranes were activated in methanol for 30 seconds, and a Mini Trans-Blot cell transfer apparatus (Bio-Rad) was prepared according to manufacturer protocol. Transfer conditions were 55V constant voltage for 2 hours at 4°C using PowerPac basic power supply (Bio-Rad). PVDF membranes were placed in 5% milk prepared in TBST, and rocked at 4°C for 1 hour. 5% milk was removed from membranes and discarded, and replaced with primary antibodies in 0.5% milk prepared in TBST. Mouse anti-Myc antibody clone 9E10 was obtained from Santa Cruz Biotechnology (Dallas, Texas) and added at a 1:500 dilution. Rabbit anti-DDDDK-tag antibody was obtained from Abcam (Cambridge, Massachusetts) and added at a 1:500 dilution. Following overnight incubation of primary antibodies gently rocking at 4°C, primary antibody was collected for re-use, and membranes were washed in TBST with vigorous shaking at room temperature, for a total of 4 washes. Membranes were placed in 0.5% milk in TBST containing anti-mouse 800CW and anti-rabbit 680LT secondary antibodies obtained from LI-COR Biosciences (Lincoln, Nebraska) and rocked at 4°C for 30 minutes. Secondary antibody was discarded, and membranes were washed 4 times as described above. Membranes were analyzed with Odyssey XF Imaging System (LI-COR) using ImageStudio software (LI-COR).

**Flow Cytometry**

Cells were centrifuged at 500 g for 5 minutes, and the cell pellet was resuspended in 100 µL of flow cytometry buffer. Cells were incubated for 30 minutes at room temperature in the dark with conjugated primary antibodies or with purified experimental proteins. Cell suspensions were subsequently diluted with 1mL of flow cytometry buffer, and centrifuged at 500 g for 5 minutes. The cell pellet was once again resuspended in 1mL of flow cytometry buffer, and centrifuged at 500 g for 5 minutes. Samples receiving primary antibody were resuspended in 400 µL of flow cytometry buffer, and held at 4°C in the dark until analysis. Samples receiving purified experimental constructs were resuspended in 100 µL of flow cytometry buffer, and incubated for 30 minutes at room temperature in the dark with conjugated primary antibodies. Suspensions were diluted, centrifuged, and washed in flow cytometry buffer as described above before being resuspended in 400 µL of flow cytometry buffer and held at 4°C in the dark until analysis. All samples, depending on experiment and cell fluorescence, received either 4′,6-diamidino-2-phenylindole
(DAPI) at a final concentration of 0.1 µg/mL, 7AAD at a final concentration of 0.1 µg/mL, or Propidium Iodide (PI) at a final concentration of 1 µg/mL to evaluate viability. Samples were analyzed on a BD Biosciences LSR II machine using BD FACSDiva software for processing of data. Data was processed using FCS Express 7 software.

**Cell Binding**

Cell binding experiments followed the flow cytometry methods above, with MOLM14, HL-60, or Jurkat cells first incubated for 30 minutes at room temperature with either FACS buffer, anti-CD33-6DMPα monomer, anti-CD3-6DMPβ monomer, or anti-CD33-6DMPα/anti-CD3-6DMPβ dimer. Following incubation and washes as described above, cells were centrifuged at 500g for 5 minutes, and incubated as described above with conjugated primary antibodies to the tags present on each component of the heterodimer. FITC-conjugated anti-myc antibody was purchased Abcam and FITC-conjugated anti-FLAG was obtained from Sigma-Aldrich (St. Louis, Missouri). Cells were washed and centrifuged as described above, and analyzed in the same way.

**Cytotoxicity**

10,000 MOLM14 or HL60 cells expressing dTomato were cultured in each well of a 96-well U-bottom plate in RPMI 1640 medium with 20% FBS and 1% P-S. CD3+ purified, Dynabead-activated T-cells were added at described effector-to-target ratios. Wells receiving purified chimeric proteins received 20 µL of PBS containing chimeric protein (ex. if 10 µg, the well receives 20 µL of 0.5 µg/µL protein; if 10 ng, the well receives 20 µL of 0.5 ng/µL protein), and complete RPMI 1640 medium was added to a final well volume of 200 µL. Cells were cultured in conditions described above for listed lengths of time. Plates were centrifuged at 500g for 5 minutes, and supernatant was discarded. Cells were washed with 200 µL of FACS buffer, centrifuged as described above, and supernatant was again discarded. Cells were resuspended in 100 µL of FACS buffer containing 0.1 µg/mL DAPI or 1 µg/mL PI, and analyzed as described above.

**Cytokine production**

The cytokine release by T cells against CD33 positive target HL60 cells and CD33 negative target Expi293 cells (ThermoFisher, Waltham MA), in presence or absence of IgGlueBiTE was determined by the U-PLEX Human Proinflammatory 7-Plex Tissue Culture Kit (Meso Scale Discovery, Rockville MD). Ten thousand of HL60 cells or Expi293 cells were plated in each well of 96-well U-bottom plate in RPMI 1640 medium with 20% FBS and 1% P-S. Dynabead-activated T cells were added at 50,000 cells per well, diluted in the same medium. The cell mixtures were then treated with IgGlue-BiTE at a range of concentrations as indicated. Cells were then cultured at 37°C with 5% CO₂ for 24 hours. The cell culture supernatant was collected, and the cytokines released by T cells were measured by MSD assay kit as instructed by the manufacturer.

**In Vivo Experiments**

All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Columbia University. NSG SGM3 mice aged 9–10 weeks were purchased from Jackson Laboratories (Bar Harbor, Maine). 0.2x10⁶ MOLM14 cells expressing luciferase and dTomato were suspended in 200 µL sterile PBS and injected into each mouse via tail vein. After two days, mice were randomly assigned into 3 treatment groups: Control group (n = 4) received PBS alone, T-cells only group (n = 4) received T-cells purified and activated as described above, and Dimer + T cells group (n = 7) received T-cells along with purified anti-CD33/anti-CD3 heterodimer purified as described above. We treated the mice at four different time points. On Day 2, control group received 200µL PBS, T-cell group received 1.0x10⁷ T-cells in 200µL PBS, and Dimer + T cells group received 100µg dimer and 1.0x10⁷ T-cells in 200µL PBS. On Day 7, control group received 400µL PBS, T-cells only group received 2.0x10⁷ T-cells in 400µL PBS, and Dimer + T cells group received 200µg dimer and 2.0x10⁷ T-cells in 400µL PBS. On Day 15 and Day 19, control group received 200µL PBS, T-cells only group received 1.0x10⁷ T-cells in 200µL PBS, and Dimer + T cells group received 100µg dimer and 1.0x10⁷ T-cells in 200µL PBS. For animal studies, a power test was used to determine the sample size needed to observe a 2-fold difference in mean between groups with 0.8 power using a 2-sided Student's t-test. Animal studies were performed without blinding of the investigator, and no animals were excluded from the analysis. Leukemia progression was monitored with bioluminescence imaging using the PerkinElmer IVIS Spectrum Optical Imaging System, beginning on Day 7 and twice-weekly thereafter. 10 minutes prior to image acquisition, mice received intraperitoneal injections of luciferase (150 µg/g body weight). To quantify leukemia progression, regions of interest were drawn around both femurs. Mice were sacrificed and analyzed when one or more leukemia-related symptoms were observed (hunched-back, significant weight loss, ruffled coat, limb paralysis). Leukemia
burden in bone marrow was measured by obtaining nucleated cells. Bone marrow was flushed from both femurs, ACK-lysed, and stained for flow cytometry analysis. The following antibodies were used to identify mouse and human cells and quantify AML burden: Bv421-CD3 (BD Biosciences, Franklin Lakes, New Jersey), APC-CD33 (BioLegend, San Diego, California), Bv510-hCD45 (BioLegend), H2k-Bv711 (BD Biosciences), Ly5-Bv711 (BioLegend), Ter119-PeCy5 (BioLegend), Propidium Iodide. AML cells were gated as PI-Ter119- hCD45 + hCD33+.

**Statistical Analysis**

Data were displayed as either individual values or mean ± S.E.M as indicated in the legends. All in vitro studies experiments were performed in triplicates. Statistical Analysis was performed using GraphPad Prism 9. Analysis of bone marrow burden and bioluminescent imaging utilized multiple unpaired t tests with Welch’s correction. Analysis of survival utilized Gehan-Breslow-Wilcoxon tests. Where appropriate, more details on statistical tests are described in the legends of this article’s figures. Experimental data meet the assumptions of the tests (normal distribution), and the variance was similar between experimental groups.

**Results**

**Design, Expression, and Purification of CD33 x CD3 Bispecific Molecule**

Several different polypeptides that form synthetic orthogonal heterodimer were described. We selected a pair referred to as 6DMP based on the available biochemical and stability data. Each component of the 6DMP (6DMPa or 6DMPb) is linked with an N-terminal single chain variable fragment (scFv). We linked, via a 3X GGGGS linker, 6DMPa with the CD33-binding scFv derived from My9-6 clone. 6DMPb was linked, via 3X GGGGS linker, with CD3-binding scFv from L2K-07 clone. We added the hinge region including 11-residue sequence with 4 cysteine residues of human immunoglobulin 2 (IgG2) on the C-terminus of both 6DMPa and 6DMPb polypeptides to allow for the formation of covalent bonds between heterodimer components once the four-helix structure forms (Fig. 1A). We named the module with 6DMP and the IgG2 hinge as IgGlue because of its ability to link the two monomer chains via a cysteine disulfide bond. The predicted structure showed a heterodimer with distinct folding of each domain as expected (Fig. 1B). Each component includes IL-2 signal peptide for secretion of the molecule, a 6xHis tag and either a c-myc-tag (CD33 targeting monomer) or FLAG-tag (CD3 targeting monomer) to enable purification and detection. Codon optimized sequence of both the monomers were synthesized and cloned in a mammalian gene expression vector. The two constructs were transfected in CHO-K1 cells either alone or together and the secreted proteins from cell culture supernatant was purified using TALON cobalt resin. Western blotting confirmed expression and purification of monomers and dimer (Fig. 1C). Exclusion of β-mercaptoethanol from Laemmli sample buffer showed high molecular weight proteins in the dimer fraction, confirming heterodimerization and suggesting covalent disulfide bridges in the IgG2-hinge region (Fig. 1D). The purified bispecific T-cell engager (BiTE) is referred to as IgGlue-BiTE in the sections below.

**Binding to Effector and Target Cells**

Functional binding efficiency was confirmed by incubating the HL-60 cell line (acute myeloid leukemia, AML) and the Jurkat lymphocyte cell line (T cell) with purified IgGlue-BiTET, followed by FITC-conjugated antibodies binding to the C-terminal FLAG-tags and myc-tags respectively. Presence of FLAG-tags on CD33 + HL-60 and myc-tags on CD3 + Jurkat cells, confirmed with FITC-conjugated secondary antibodies, indicates that CD33-scFv and CD3-scFv binding is maintained while the IgGlue-BiTET remains intact (Fig. 2). Antibodies to myc and FLAG tags do not bind cells in the absence of IgGlue-BiTET or appropriate monomer. CD33-binding monomer alone does not bind to Jurkat, and CD3-binding monomer alone does not bind to HL-60 confirming the specificity of the IgGlue-BiTET (Fig. 2C&D, Supplementary Fig. 1, 2&3).

**In vitro Cytotoxicity by Effector Cells on Target Cells**

To establish the functional activity of the IgGlue-BiTET in vitro, we selected CD3 + T cells from human peripheral blood mononuclear cells. The purified T cells were activated using CD3/CD28 Dynabeads. Activated T cells were then co-cultured with AML cell lines HL-60 (Fig. 3A&B) and MOLM14 (Fig. 3C&D). AML cell lines were cultured alone or in a 1:5 ratio with T cells, with or without IgGlue-BiTET, and incubated for 16 hours. The cytotoxicity was evaluated by flow cytometry based assay with gating...
strategy described in Supplementary Fig. 4 to identify live and dead AML cells. Both monomers were non-toxic to AML in both the absence or presence of T cells (Supplementary Fig. 5), and IgGlue-BiTE alone was similarly non-toxic (Fig. 3B&D). T cells alone had some AML cytotoxicity, but adding IgGlue-BiTE bolstered AML cytotoxicity in a dose-dependent manner (Fig. 3B&D). This effect was stronger in MOLM14 than in HL-60, consistent with reported CD33 expression levels of these cell lines but we cannot rule out other unknown factors. These results indicate IgGlue-BiTE can simultaneously bind CD33-expressing AML and CD3-expressing cytotoxic T cells and facilitate T cell mediated cytotoxicity, and that the IgGlue-BiTE itself is not toxic.

In vitro IgGlue-BiTE Mediated Cytokine Production by T cells in the Presence of Target Cells

To evaluate if robust T cell activation being achieved, T cells were incubated with various amounts of IgGlue-BiTE in the absence of target cells, or in presence of AML cells (HL-60) or Expi293 cells (negative control, no CD33 expression). Cytokine production into the culture supernatant was measured using U-Plex MSD kit (MSD, Maryland). As expected, there was a robust expression and release of various cytokines including IFNg, TNFa, GM-CSF, IL-2, IL-6, and IL-10. No cytokine production was observed in T cells alone or with a CD33 negative target suggesting a IgGlue-BiTE mediated robust T cell activation in a target specific manner (Fig. 4).

In vivo Tumor Suppression by IgGlue-BiTE

To establish the functional activity of the IgGlue-BiTE in vivo, we utilized a previously reported AML model of post-remission with minimal residual disease, injecting MOLM14 cells expressing luciferase in NSG-SGM3 mice and allowing two days for engraftment. Mice received initial injections on Day 2 and Day 7 of either PBS, or T cells incubated with two different doses (100µg or 200µg) of IgGlue-BiTE, and further injections on Day 15 and Day 19 of PBS, T cells, or T cells incubated with 100µg IgGlue-BiTE respectively (Fig. 5A). Whole-body bioluminescent imaging (BLI) showed no detectable AML burden in all 3 groups at Day 7 but progressed more slowly in the group receiving T cells with IgGlue-BiTE at Day 10 and Day 13 (Fig. 5B&C). By Day 16, mice in the control and T cell only groups began showing terminal symptoms of leukemia such as hind-limb paralysis. Following the final injection on Day 19, luciferase signal in the bone marrow is reduced in those mice receiving the combination of T cells and IgGlue-BiTE (Fig. 5C and Supplementary Fig. 6) by day 21 and all mice in control or T cells only groups were deceased (Fig. 5D). In mice
treated with T cells incubated with IgGlue-BiTE, significant growth of solid extramedullary tumors was observed through day 21 to day 63 (Supplementary Fig. 6). Despite the mice in this group were sacrificed due to solid tumor burden or clinical symptoms, a marked improvement in survival was observed for T cells/IgGlue-BiTE group. For all experimental groups, AML burden in the bone marrow was evaluated with flow cytometry at the time of death (Fig. 6). No AML signal was detected in the bone marrows isolated from T cells/IgGlue-BiTE group, suggesting a complete clearance of AML from the bone marrows. These results together suggest that this IgGlue-BiTE format can bolster the regression of AML in a mouse model of relapse, and that such an approach may be capable of eliminating AML from the bone marrow to considerably extend survival.

Discussion

There are over 20 technology platforms that are currently in commercial use for the development and production of BsABs and many suffer manufacturing challenges including the expression and purification of the desired product to homogeneity. The first BsABs were produced by fusing two different hybridoma resulting in random association of heavy and light chains resulting in reduced fraction of desirable product. To address these production issues, other technologies such as knobs-into-holes, strand-exchange engineered domain, and electrostatic steering, CrossMab and Orthogonal Fab were developed. The advent of designed chimeric proteins, particularly those with multiple antigen-binding regions, has introduced numerous questions about steric hindrance and optimal N- and C-terminal orientations of the components of a fusion protein. We reason that a structure composed of two polypeptides, such as the elegant orthogonal heterodimers reported by the Baker group, could be utilized to overcome issues of steric hindrance, and ensure that antigen-binding regions are not denied the more favorable N-terminus. BiTEs with orientations that must sacrifice the potency of an antigen-binding region may benefit from this approach. Future studies should confirm whether this structure maintains or even improves the potency of favored orientations for both antigen-binding regions compared to conventional N-to-C oriented single-chain BiTEs, and what the effects may be on bioavailability in vivo.

In this study, we designed a new type of bispecific protein by fusing scFvs (anti-CD33 and anti-CD3) to a set of synthetic polypeptides that prefer a heterodimer conformation. Chen et al used Crick equations to model four-helix structures with hydrogen bond networks that imitate DNA base pairing and identified a set of polypeptides that are expressed exclusively as heterodimers—without homodimers, and without tetramers or larger aggregates. Adding the IgG2 hinge region to the C-terminus of these polypeptides (IgGlue) ensured a covalent linkage between the monomer resulting in a stable heterodimer. Using biochemical approaches, we showed that this type of molecule exists as a covalently-linked heterodimer, retaining the ability to bind both CD33 and CD3 without dissociating and without inappropriate scFv binding to off-target cells. This binding was confirmed to be functional, facilitating killing of AML by T cells in vitro in a dose-dependent manner. Our in vitro data also showed that this IgGlue-BiTE is not itself toxic to AML, and that its monomeric components are similarly not toxic. The heterodimer toxicity is dependent on simultaneous binding to CD33 and CD3, as monomeric components do not facilitate T cell cytotoxicity. This approach translated to an in vivo model of AML with minimal residual disease, eradicating AML from the bone marrow of mice receiving T cells loaded together with IgGlue-BiTE and extending survival of these mice compared to those receiving T cells alone. This approach may be able to rescue organisms from high levels of AML burden, as whole-body bioluminescence in the IgGlue-BiTE group of mice approached fatal levels before eradication. Future studies should titrate the dose and dosing schedule to better understand minimum effective doses. Administration of IgGlue-BiTE separately from T cells, rather than with ex vivo incubation, will also better inform the potential clinical application of such a bispecific antibody.
We reason that this particular approach to design heterodimeric molecules may be applicable to numerous target- and effector-cell combinations. NK cell cytotoxicity, for example, is an appealing tool underutilized by existing therapies. While this can be paired with antigen-ablation techniques previously described, such as ablation of CD33 from healthy progenitor cells, it would be most appealing where cancer-specific antigens exist and may be particularly potent in combination with immune checkpoint inhibitors. Beyond the diversity of malignancies that may be explored with this bispecific format, trispecific or polyspecific molecules may be possible by chaining multiple heterodimer-forming domains and introducing additional functional components of interest.

**Declarations**

**Acknowledgments**

Research reported in this publication was performed in the CCTI Flow Cytometry Core, supported in part by the Office of the Director, National Institutes of Health under awards S10RR027050 (LSRII) and S10OD020056 (Influx sorter). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Mice imaging were performed using instrumentation maintained by the Cancer Center Small Animal Imaging Shared Resource with NIH grant #P30 CA013696 (National Cancer Institute).

**Author Contributions**

AB, FB, AMA, MC, and SM participated in the design of the experiments; AB, FB, MC, JD, and XD performed experiments. AB, FB, XD, JD, AMA, MC, and SM analyzed and interpreted data. AB, AMA, AMG, and SM wrote the manuscript. FB, XD, PD, MC, and JD edited the manuscript. SM conceived the idea. AMA and SM participated in the design of the study. All authors reviewed and checked the final version of the paper.

**Competing interests**

Columbia University has granted an exclusive option to Brahma Therapeutics to license technology that is the subject of this study. A.B., F.B., A.M.A., and S.M. are co-inventors on pending patent applications filed by Columbia University and optioned to Brahma Therapeutics. S.M. has equity ownership and is on the Scientific Advisory Board of Brahma Therapeutics. M.C., J.D. and P.D. is an employee of Brahma Therapeutics.

**Data Availability Statement**

All data relevant to the study are included in the article or uploaded as supplementary information.

**References**


**Figures**
Figure 1

Design, predicted structure, in vitro expression and purification of IgGLUE-BITE. A Schematic drawing of various components of each monomer. B Two views (side and top) of predicted structure of the dimer using AlphaFold multimer program and visualized using ChimeraX. C Western blot of cell culture supernatants before and after 6xHis purification with cobalt resin. D Western blot of purified heterodimer prepared in reducing (+) and non-reducing (-) conditions.
Figure 2

*In vitro binding of IgGLUE-BiTE with AML (HL-60) and T-cell (Jurkat).* Representative flow plots of CellTrace Violet stained HL60 AML cells (A) or Jurkat cells (B) incubated for 30 min with 1 µg BiTE. A dot plot showing percent binding of 1 µg monomers or dimer with CD33+ HL-60 (C) and CD3+ Jurkat cells (D). The box around the plus sign indicated these samples had dimers. Data reported as individual values from triplicates.
**Figure 3**

*In vitro cytotoxicity of T-cells mediated by IgGLUE-BiTE.*  
A Representative flow plots of dTomato-expressing HL-60 AML cells treated with heterodimer and CD3/CD28-activated T cells.  
B Specific cytotoxicity on HL-60 cells.  
C Representative flow plots of dTomato-expressing MOLM14 AML cells treated with heterodimer and CD3/CD28-activated T cells.  
D Specific cytotoxicity on MOLM14 cells specific cytotoxicity considered as (CD33+ killed – CD33+ killed by T cells alone) / (100 – CD33+ killed by T cells alone). All conditions incubated 16hr. Data reported as individual values from triplicates.
Figure 4

**IgGlue-BiTE mediated T-cell cytokine production.** T cells were incubated with indicated amounts of IgGlue-BiTE in the absence or presence of AML cells (HL-60) or Expi293 and cytokine secretion was measured using ELISA. Data reported as mean ± S.E.M.
IgGLUE-BITE facilitates T cell clearance of AML from bone marrow in vivo and extends survival. A Schematic of the study design: Mice received intravenous injections of $0.2 \times 10^6$ MOLM14 cells expressing luciferase. At subsequent listed days, mice received injections of PBS, activated T cells, or activated T cells bound to heterodimer at listed concentrations. Mice were imaged (IVIS BLI) or bone marrow harvested to assay tumor burden or clearance. B Whole-body bioluminescent imaging was used to monitor AML progression 1-2 times per week. Whole-body bioluminescent images at listed days. Background removed according to total flux from uninjected imaging control (*). Additional IVIS BLI images for days 21, 23, 27, 31 and 33 are provided in supplemental figure 6. C Total flux (photons/s) taken from uniform region of interest including hind limbs and abdomen. Data reported as total flux from individual mice and color coded based on the treatment group. The day mice were treated is indicated by an arrow head pointing down. D Kaplan-Meier curves. Mice were euthanized upon exhibiting signs of leukemia or symptoms such as rapid weight loss, hunching, reduced mobility, paralysis. Tick marks indicate mice that died during treatment procedure.
Figure 6

No evidence of tumor in bone marrow aspirates. A Top: gating scheme used to quantify AML burden in bone marrow. Bottom: Flow dotplots of AML cells gated as Ter119- human CD45+ mouse Ly5/H2kd- CD33+. B AML burden quantified as total fraction of CD33+ cells among all Ter119-CD3- (**: p<0.01. ****: p<0.0001. Unpaired t-test with Welch’s correction). Data reported as individual values from each mice.

Supplementary Files

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- SupplementalFigureR.docx