A CS1-NKG2D Bispecific Antibody Collectively Activates Cytolytic Immune Cells against Multiple Myeloma

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Abstract

Multiple myeloma (MM) is an incurable hematologic malignancy of plasma cells, with an estimated 30,000 new cases diagnosed each year in the United States, signifying the need for new therapeutic approaches. We hypothesized that targeting MM using a bispecific antibody (biAb) to simultaneously engage both innate and adaptive cytolytic immune cells could present potent antitumor activity. We engineered a biAb by fusing an anti-CS1 single-chain variable fragment (scFv) and an anti-NKG2D scFv (CS1-NKG2D biAb). Although NKG2D is a potent activation receptor ubiquitously expressed on mostly cytolytic immune cells including NK cells, CD8+ T cells, and NKT cells, the CS1 tumor-associated antigen on MM represents a promising target. CS1-NKG2D biAb engaged human MM cell lines and NKG2D+ immune cells, forming immune synapses. In effector cells, CS1-NKG2D biAb triggered the phosphorylation of AKT, a downstream protein kinase of the activated NKG2D–DAP10 complex. The EC50 values of CS1-NKG2D biAb for CS1high and for CS1low MM cell lines with effector PBMCs were 10−12 and 10−9 mol/L, respectively. CS1-NKG2D biAb acted through multiple types of immune cells, and this induced cytotoxicity was both CS1- and NKG2D-specific. In vivo, survival was significantly prolonged using CS1-NKG2D biAb in a xenograft NOD-SCID IL2re−/− (NSG) mouse model engrafted with both human PBMCs and MM cell lines. Collectively, we demonstrated that the CS1-NKG2D biAb facilitated an enhanced immune synapse between CS1+ MM cells and NKG2D+ cytolytic innate and antigen-specific effector cells, which, in turn, activated these immune cells for improved clearance of MM. Cancer Immunol Res; 6(7); 776–87. ©2018 AACR.

Introduction

Multiple myeloma (MM) is an incurable malignant disease of plasmacytes, with an estimate of 30,770 new cases diagnosed in 2018 (1). Current therapeutics for MM include proteasome inhibitors (bortezomib and carfilzomib), immunomodulatory agents (thalidomide, lenalidomide, and pomalidomide), and stem cell transplantation (2). Both daratumumab, an antibody against CD138, and elotuzumab, an antibody against CS1 (SLAMF7), exert their anti-MM activity, at least in part, through enhanced Fc receptor-mediated cytotoxicity provided by both natural killer (NK) cells and phagocytes (3–5). Elotuzumab, when used together with lenalidomide, dexamethasone, or bortezomib, has demonstrated improved clinical outcomes for refractory MM compared with therapy without elotuzumab (6). On the other hand, lenalidomide may exert its anti-MM activity, in part, through modulation of both T and NK cells (7, 8).

NKG2D is a lectin-type receptor and one of the major activating receptors (biAb) and T/NK-cell engager (BiTE/BiKE) therapies fall into this new class of treatment, with the first BiTE targeting CD19 and CD3 having been FDA approved in 2014 (16). Expanding the discovery and characterization of tumor-associated antigens explores the possibility of applying this antibody platform to other cancers.

MM cells express high CS1 (CD319 or SLAMF7), a surface lymphoctic activity molecule, whereas NK cells and a subset of activated T cells have low expression (17). Its expression, as determined by staining with the CS1 monoclonal antibody (mAb) elotuzumab, is found on nearly all tumor cells in 95%
of MM patients and has become a target for therapy with elotuzumab (2, 18). However, unless combined with other chemotherapies such as lenalidomide, dexamethasone, or proteasome inhibitor bortezomib, elotuzumab has no significant clinical benefits to MM patients (3). Our group reported that anti-CS1 chimeric antigen receptors (CAR) T and NK cells could effectively eradicate MM cells (19, 20). This suggests that targeting CS1 while activating T or NK cells remains a promising therapeutic strategy for MM.

Here, we describe the generation and therapeutic activity of a biAb targeting CS1 on MM cells and NK2GD on cytototoxic immune effector cells. This CS1-NK2GD biAb was derived from clones of high-affinity anti-CS1 (20) and anti-NK2GD (21). We hypothesized that by engaging CS1 on MM and simultaneously activating NK2GD on all NK2GD-expressing immune cells, including NK cells, CD8+ T cells, γδ T cells, and NKT cells, we could exert an additive cytototoxic effect against MM cells. Our in vitro data demonstrated that CS1-NK2GD biAb induced a dose-dependent increase in specific cytotoxicity of these effector cells against CS1+ MM cells, as well as IFNγ production, and significantly prolonged survival when administered in vivo to an NOD-SCID Ig226–/– (NSG) mouse model of human MM.

Materials and Methods

Bi-specific antibody construction, expression, and purification

CS1-NK2GD biAbs were in silico designed (Fig. 2A), and single chain variable fragments (scFv) of the mouse anti-human NK2GD (21) and anti-human CS1 (19) mAbs were joined with non-immunogenic human muscle aldose protein linker (22). A six-histidine tag was added to the c-terminus of anti-CS1 scFv. A secretory signal peptide H7 was added in front of the entire sequence (23). The sequence was then codon-optimized (22), synthesized, and subcloned into a lentiviral vector pCDH-CMV-MCS-EF1α-GFP (SBI Bioscience). The lentivirus generated was used to transduce a CHO-S cell line (Invitrogen), which was authenticated by the manufacturer, passaged three times, and authenticated since receipt but were not passaged for more than 2 months. All the cell lines used were routinely tested for mycoplasma using MycoAlert PLUS Mycoplasma Detection Kit fromLonza.

Peripheral blood of 48 healthy donors and 3 MM patients were obtained from American Red Cross and The James Cancer Hospital, respectively. The study was approved by the Institutional Review Board at The Ohio State University and was conducted in accordance with the Declaration of Helsinki. Informed and written consents were obtained from all 3 patients, and no inclusion/exclusion criteria were specified for the study. Human peripheral blood mononuclear cells (PBMC) were isolated from the healthy donors or MM samples within 6 hours after receipt by Ficoll-Paque Plus (GE Healthcare Life Science) gradient density. Human NK cells from peripheral blood were negatively enriched using a RosetteSep human NK cell enrichment cocktail (catalog no. 15065, Stem Cell Technology), whereas human T cells were enriched using a RosetteSep human T-cell enrichment cocktail (catalog no. 15061, Stem Cell Technology), following the manufacturer’s instructions. In indicated experiments, CD56- or CD3-negative cells were collected from the flow-throughs of the PBMCs stained with CD56 or CD3 microbeads (Miltenyi Biotech), respectively. To test the specificity of CS1-NK2GD biAbs, NK2GD-negative cells were sorted from IL2 (500 U/mL)-primed PBMCs.

Antibodies and flow cytometry

Antibodies used in this study include from BD Biosciences: anti-human CD3 (clone SK7, FITC, and PE-CF594-conjugated), anti-human CD4 (clone RM4-5, FITC-conjugated), human anti-CD8 (clone RPA-T8, FITC-conjugated), anti-CD56 (clone N901, APC-conjugated), anti-CD14 (clone MφP9, FITC-conjugated), anti-human CD45 (clone 2D1, APC-H7-conjugated), anti-mouse CD45 (clone 30-F11, PE-conjugated), anti-human NK2GD (clone 1D11, PE-conjugated), anti-human TCRγδ (11F2, FITC-conjugated), anti-human CD19 (clone SJ25C1, FITC-conjugated), anti-human CD20 (clone L27, FITC-conjugated), anti-human CD34 (clone 8G12, APC-conjugated); from BioLegend: anti-mouse CD3 (clone 145-2C11; PE-conjugated), anti-phallolidin (APC-conjugated), and anti-human TCRVß24-Int18 (clone 6B12, APC-conjugated); from Miltenyi Biotech: anti-CD38 (clone REA572, PE-Vio770-conjugated); from Thermo Fisher Scientific: anti-CD319 (CS1/SLAMF7; clone 162, PE-conjugated), and anti-human CD138 (clone DL-101, APC-conjugated). For staining NK2GD ligands, a recombinant NK2GD-Fc chimera protein was used (catalog

SDS-PAGE and Coomassie brilliant blue staining under the standard procedure to determine the presence of monomers and dimers. The eluted fractions containing monomer (CS1-NK2GD biAb or control biAb) fractions were sequentially dialyzed at pore size cutoffs of 100 and 50 kDa against PBS before use in in vitro or in vivo studies. Purified biAbs were routinely analyzed by SDS-PAGE and stained with Coomassie brilliant blue for size estimation and quality control.

Multiple myeloma cell and cell isolation

The multiple myeloma cell lines MM.1S, NCI-H929, and RPMI-8226 were obtained from ATCC and cultured under the manufacturer’s instruction. U266, L363, and OPM2 (from Dr. Don Benson) were cultured in RPMI 1640 with 10% FBS and 1% antibiotic antimycotic (Invitrogen). These cell lines have not been authenticated since receipt but were not passaged for more than 2 months. All the cell lines used were routinely tested for mycoplasma using MycoAlert PLUS Mycoplasma Detection Kit from Lonza.

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number 1299-NK-050, R&D Systems). Anti-human MICA/B and human IL1BP2 antibodies were from R&D Systems and both were PE-conjugated. To stain, 1 × 10⁶ cells were first washed once with PBS, then stained with antibodies in staining buffer (PBS with 0.5% BSA and 2 mmol/L EDTA) for 20 minutes at room temperature. All samples were analyzed with an LSRII flow cytometer (BD Biosciences).

Identification of immune effector cell subsets and hematopoietic stem cells

Total T cells in PBMCs were stained with antibodies directed against either CD3 alone, CD3 and CD4, CD3 and CD8, and CD3 and TCRVγδ for γδT cells and CD3 and CD56 for total NKT cells. NK cells were identified as CD56⁺CD3⁻. Invariant (i) NKT cells were stained with antibodies directed against CD3 and TCRVα/β-Jε18. Monocytes/macrophages were identified as CD14⁺CD16⁻. Hematopoietic stem cells were identified as CD34⁺CD38⁻. Hematopoietic stem-like cells were identified as CD34⁺Lin⁻. Lin⁺ was defined using a cocktail of monoclonal antibodies directed against CD3, CD56, CD19, and CD20.

Flow-based cytotoxicity assay

The flow-based cytotoxicity assay was performed as previously described (24). Briefly, the multiple myeloma cell lines MM.1S, NCI-H929, and RPMI-8226 were transduced with a pCDH-GFP vector (System Biosciences) to establish corresponding target cell lines stably expressing GFP. For U266, OPM2, and L363 cells, they were labeled with Calcein-AM (BD Biosciences) as per the manufacturer’s instructions. Briefly, 1 × 10⁶ target cells were labeled with 5 μmol/L Calcein-AM for 30 minutes at 37°C water bath protected from light. The cells were then washed for 4 times with RPMI 1640 medium supplemented with 10% FBS before being used as target cells. The target cells were then cocultured with IL2 (500 U/mL)-primed PBMCs at an effector-to-target (E:T) ratio of 20:1, 10:1, 5:1, and 1:1 for 4 hours in RPMI 1640 medium supplemented with 10% FBS. The cells were then harvested for flow cytometry analysis of induced cytotoxicity. Calcein-AM-labeled autologous PBMCs were also used as target cells and cocultured with autologous IL2-primed PBMCs at an E:T ratio of 10 in the presence of biAbs.

To test the effect of soluble NKG2D ligands or CS1 antigen on the cytotoxicity, 5 μg/mL NKG2D-Fc or 10 ng/mL recombinant CS1 protein (Biomatik) was added to the coculture of the IL2-primed PBMCs, MM.1S, and biAbs. To test the specificity of the CS1-NKG2D biAb on the cytotoxicity, NKG2D-neutralizing antibody (10 μg/mL, clone 1D11, BD Biosciences) was used to pretreat the effector cells before coculturing with target cells in the presence of biAbs. For cytotoxicity assay using primary MM patient sample as target, MM patient PBMCs were cocultured with allogeneic PBMCs from healthy donors at an E:T ratio of 10:1 in the presence of CS1-NKG2D biAb or control biAb for 24 hours. Cells were then stained with antibodies against CD38 and CD138. The CS1bright subset in MM patient samples was identified in the CD38hi/CD138dim/+ population. Dose of biAb used was 50 μg/mL in most cytotoxicity experiments, except when particularly specified.

Imaging flow cytometric detection of immune synapse

Isolated total CD3⁺ T or NK cells were activated at 1 × 10⁶ cells/mL with IL2 (500 U/mL) overnight. The cells were harvested and labeled with CD3 or CD56 PE-conjugated monoclonal antibodies. These labeled effector cells were cocultured with GFP-transduced-MM.1S MM target cells at 1:1 E:T ratio for 30 minutes in the presence of PBS, control biAb (100 μg/mL), or CS1-NKG2D biAb (100 μg/mL). After coculture, cells were fixed with 2% paraformaldehyde in PBS (Millipore Sigma) for 20 minutes on ice, permeabilized with PBS containing 0.5% saponin (Millipore Sigma) and 2% FBS for 20 minutes on ice. The cells were washed and stained with Alexa Fluor 647-phalloidin and DAPI (Thermo Fisher Scientific) for 20 minutes on ice. All the samples were washed twice and resuspended in 50 μL PBS.

The cells were analyzed with Amnis ImageStream X Mark II imaging flow cytometer, and the data were analyzed with IDEAS (Amnis). Briefly, single cells were identified based on the cell size, focus attributes, and nucleus DAPI positivity stain. An actin valley mask was then created and defined in the colocalization of two cells (GFP and PE positive) by a high local pixel intensity formed with accumulation of phalloidin staining from actin in the immune synapse. A valid synapse was defined by high bright detail similarity score (BDS) and high bright details intensity (BDI) on the actin valley mask images. BDS was calculated based on the spatial location of the image pairs and determined the degree of overlapping between the two images. BDI computed the intensity of bright spots that were 3 pixels in radius or less. A plot of BDS versus BDI identified the valid synapses, and the relative frequency of synapses formed in indicated conditions was calculated.

Immunoblotting

Primary NK cells (1 × 10⁶) were stained with either CS1-NKG2D biAbs (100 μg/mL) or anti-NKG2D (BD Bioscience) for multiple time periods in a 37°C incubator. Cells were then harvested at indicated time points. The cells were lysed for 10 minutes on ice in a mammalian cell lysis buffer (Thermo Fisher Scientific) supplemented with a protease inhibitor cocktail (cOmplete, Millipore Sigma), 1 mmol/L sodium orthovanadate, and 1 mmol/L PMSE. The protein lysates were quantified using a BCA protein assay kit (Thermo Fisher Scientific). Thirty micrograms of total protein was resolved with SDS–PAGE and transferred to the nitrocellulose membrane of a Trans-Blot Turbo transfer system (Bio-Rad Laboratories) using a Trans-Blot Turbo Transfer System (Bio-Rad). After the membrane was blocked with Odyssey blocking buffer/PBS (Li-Cor Biotechnology), it was immunoblotted with a rabbit anti-human phospho-AKT antibody (clone D9E, Cell Signaling) and a mouse anti-human total β-actin (clone L44; Santa Cruz Biotechnology) as the loading control. A donkey anti-mouse IgG (H+L) IRDye 680RD antibody (Li-Cor) and a goat-anti-rabbit IgG (H+L) IRDye 680RD antibody (Li-Cor) were used as secondary antibodies. The stained membrane was washed 4 times with PBS plus 1% Tween 20 and 2 times with PBS alone before it was scanned with an Odyssey CLx imaging system (Li-Cor). The expression of the phospho-AKT was normalized with that of the loading control.

ELISA

To determine the IFNγ production of the biAb-induced PBMCs, cocultures of MM.1S (1 × 10⁶) and IL2-primed PBMCs...
Figure 1. NKG2D, NKG2D ligands, and CS1 expression. A, Box-and-whisker plot of percentage of NKG2D⁺ cells in peripheral blood immune cells. Data represent the median percentage among 5 healthy donors. B, Expression of NKG2D on T cells, CD56⁺ NKT cells, and NK cells at rest or overnight IL2-activated conditions. Results are mean MFI ± SEM from three independent experiments. *, P < 0.05; **, P < 0.001 using the Student t test. C, The expression NKG2D ligands (white) on MM cell lines stained with NKG2D-Fc antibody compared with the isotype (gray). Numbers on the top right corner: mean fluorescent intensity (MFI). Results shown are representative of three independent staining experiments. D, NK cell natural cytotoxicity against MM.1S, NCI-H929, RPMI-8226, OPM2, and L363 cells. Target cells were coincubated with NK cells at indicated E:T ratios for 4 hours. Results are mean specific lysis with SEM from three independent experiments and 3 healthy donors. E, Expression of CS1 on different MM cells displayed as histograms compared with isotype. Numbers on the top right corner: MFI. Results shown are representative of three independent staining experiments.
Cell-free supernatants were harvested after 48 hours, and the concentration of human IFNγ in the culture supernatants was quantified in ELISA assays with an IFNγ antibody pair (clones XMG1.2 and 2G1, Thermo Fisher Scientific). For quantifying the biAb kinetics along time from different routes of administration in vivo, RAG2Il2r−/− mice were used. Briefly, 20 μg CS1-NKG2D biAb was injected into the mice via intravenous, intraperitoneal, or subcutaneous routes. Blood (100 μL) was collected from tail veins at indicated time points over 48 hours. Cell-free plasma samples were collected by centrifugation the blood samples. An in-house biAb ELISA was constructed to quantify the concentration of biAb. Briefly, biAb-containing plasma samples were first coated onto a 96-well ELISA plate (Corning) at room temperature for 2 hours. A His-tagged protein (Biomatik) with known concentration was used as a standard. The plate was blocked with a PBS buffer containing 1% BSA and 0.05% Tween 20, washed with PBS supplemented with 0.05% Tween 20, and incubated with HisProbe-HRP conjugate (1 μg/mL; Thermo Fisher Scientific) for 15 minutes. The plate was then washed, incubated with 100 μL per well tetramethylbenzidine substrate (Agilent Technologies) for 15 minutes and read at 450 nm using a Synergy HT microplate reader (Biotek).

**In vivo experiment**

All the animal studies were approved by The Ohio State University Institutional Animal Care and Use Committee. Eight to 12-week-old NSG mice bearing a xenograft of MM.1S cells were used as a preclinical model to determine the efficacy of the CS1-NKG2D biAb. Mice were X-ray irradiated at 100 cGy the day before 5 × 10⁶ MM.1S were intravenously (i.v.) injected. The next day, 5 × 10⁶ IL2-primed PBMCs were injected intraperitoneally. Human IL2 (100 U/mouse) was subcutaneously injected starting 1 day after effector cell injection and every other day for 2 weeks. CS1-NKG2D biAbs or control biAbs (200 μg/kg) were subcutaneously injected at the flank to the corresponding group twice a day for 2 weeks starting 1 day after effector cell injection. The progression of the disease was monitored by assessing body condition score (BCS) every day, and survival data were recorded. The mice were sacrificed when they reached an endpoint at BCS<2, paralysis, or 20% body weight loss.

**Statistical analysis**

For data following normal distribution, or normal after transformation, Student t test or paired t test was used to compare two independent groups or two matched groups. Linear model was used to compare multiple independent groups. Linear mixed model was applied to multiple group comparisons under the variance-covariance structure due to repeated measures. Survival data, the Kaplan–Meier method was applied to estimate survival function, and the log-rank test was used to compare the survival between two groups. P values were adjusted for multiple comparisons by the Holm procedure. A P value of 0.05 or less was considered statistically significant. All data were analyzed with R version 3.3.1 and SAS 9.4.

**Results**

**Surface expression of NKG2D and its ligands on MM cells**

As previously reported (25), we found that NK cells, CD8⁺ T cells, 87 T cells, CD56⁺ NKT cells, and TCRvα24-Jε18⁺ INKT cells had high surface density expression of NKG2D (Fig. 1A). In contrast, CD14⁺LIN macrophage and CD34⁺ stem cells had low/no expression of NKG2D. Expression of NKG2D on CD56⁺ NKT cells and NK cells could be significantly increased in the presence of IL2 (Fig. 1B). The susceptibility of MM cells to NK2D-mediated NK cell cytotoxicity relies on their expression of NKG2D ligands, namely, MICs or ULBPs (14). We found that five of six human MM cell lines had low or absent expression of MICs or ULBPs (Fig. 1C). This likely contributes to the relative resistance to NK cell natural cytotoxicity seen in these cell lines (Fig. 1D). Because the NKG2D receptor remains one of the major activation receptors for cytolytic effector cells, we attempted to trigger NKG2D activation on the NKG2D⁺ cytolytic immune cells and simultaneously engage MM cells via the MM-associated antigen, CS1. We, therefore, examined CS1 expression on the six MM cell lines. Each showed different surface density expression of CS1, with MM.1S, OPM2, and L363 showing high expression, H929 showing...
intermediate expression, and RPMI-8226 and U266 showing low expression (Fig. 1E).

A CS1-NKG2D biAb retargeting CS1 and NKG2D

We designed the CS1-NKG2D biAb in silico. It consisted of two codon-optimized scFv from a CS1 mAb (20) and an NKG2D antibody (21) joined together by a nonimmunogenic protein linker derived from human muscle aldose (26). Each scFv contained a corresponding heavy chain (VH) and light chain (VL) connected by a 15-amino acid long glycine-serine (GS) linker. The entire fragment was led by an IgG secretion signal peptide H7 (23) and expressed in a lentiviral vector (Fig. 2A). Culture supernatants from the stable CHO-S cell line carrying the biAb were purified as described. A typical elution profile is shown (Fig. 2B). A similar biAb with a CDR3 deletion on the VH of anti-NKG2D scFv was produced as our negative control biAb (termed control biAb hereafter; Supplementary Fig. S1). SDS–PAGE showed the CS1-NKG2D and control biAbs had the correct sizes of 56 and 50 kDa, respectively, as expected from computational analysis (Fig. 2C).

Using ImageStream imaging flow cytometry, we confirmed that functional immune synapses were formed in the presence of CS1-NKG2D biAb but not control biAb (Fig. 3A and B). A 1.92-fold increase in antigen-specific immune synapses was observed in cocultures of NK cells and MM cells in the presence of CS1-NKG2D biAb but not with the control biAb (Fig. 3C).

Triggering NKG2D on NK cells by CS1-NKG2D biAb induces phosphorylation of AKT

To determine the activation induced by the CS1-NKG2D biAb, we incubated IL2-primed NK cells with full-sized anti-NKG2D as a positive control or CS1-NKG2D (right). MM.1S MM cells: GFP; NK cells: PE; nuclei: DAPI; and actin: phalloidin. Cell-to-cell conjugation shown in merged GFP and PE detection channels. Valid synapses were identified only by colocalization of actin (Alexa Fluor 647) at the interface of MM.1S MM (GFP) and NK (PE) in the merged image of the 3 detection channels (red arrows). B, Dot plots of immune synapses formed between MM.1S and NK cells in the presence of control biAb (left) and CS1-NKG2D (right). BDS: degree of overlapping between the two images of effector and target cells. BDI: intensity of bright spots that were <3 pixels in radius. Blue gates: immune-synapses (high BDS and high BDI). Pink gates: low BDS and low BDI formed by coincidence. C, Fold increase in immune synapse formations between the MM.1S MM and NK cells in the presence of control biAb or the CS1-NKG2D biAb. Total percentage of synapses from control biAb was normalized as 1. Data are mean ± SD from two independent experiments. **, P < 0.01 using Student t test.

Figure 3.

Immune synapses between the CS1+ MM.1S MM cells and NK cells increase in the presence of CS1-NKG2D biAb but not control biAb. A, Representative snapshot images of each conjugation between 1 × 10^6 MM.1S MM cells and 1 × 10^5 NK cells in the presence of 100 μg/mL control biAb (left) or CS1-NKG2D (right). MM.1S MM cells: GFP; NK cells: PE; nuclei: DAPI; and actin: phalloidin. Cell-to-cell conjugation shown in merged GFP and PE detection channels. Valid synapses were identified only by colocalization of actin (Alexa Fluor 647) at the interface of MM.1S MM (GFP) and NK (PE) in the merged image of the 3 detection channels (red arrows). B, Dot plots of immune synapses formed between MM.1S and NK cells in the presence of control biAb (left) and CS1-NKG2D (right). BDS: degree of overlapping between the two images of effector and target cells. BDI: intensity of bright spots that were <3 pixels in radius. Blue gates: immune-synapses (high BDS and high BDI). Pink gates: low BDS and low BDI formed by coincidence. C, Fold increase in immune synapse formations between the MM.1S MM and NK cells in the presence of control biAb or the CS1-NKG2D biAb. Total percentage of synapses from control biAb was normalized as 1. Data are mean ± SD from two independent experiments. **, P < 0.01 using Student t test.
the CS1-NKG2D biAb, whereas the signals from CS1-NKG2D biAb diminished at 30 minutes (Fig. 4). This demonstrated that the CS1-NKG2D biAb could bind to and trigger activation of effector target cells via NKG2D.

**CS1-NKG2D biAb induces cytotoxicity and IFNγ production of PBMCs**

To assess the functionality of the CS1-NKG2D biAb, we cocultured IL2-primed PBMCs as effectors with MM cell lines MM.1.S, H929, or RPMI-8226 as targets, representing MM cells with high, intermediate, and low CS1 expression, respectively. Dose-dependent increases in specific lysis were observed in the presence of CS1-NKG2D biAb but not in the presence of RPMI-8226 or the negative control biAb (Fig. 5A). The EC_{50} for the CS1^{high} MM.1.S, CS1^{int} H929, and CS1^{low} RPMI-8226 MM cell lines were 10^{-12} mol/L, 10^{-11} mol/L, and 10^{-9} mol/L, respectively. Primary MM patient peripheral blood samples were also used to validate the efficacy of the CS1-NKG2D biAb. All three samples contained the CS1^{bright} subset in the CD38^{bright}CD138^{dim/−} MM population (Supplementary Fig. S2). A reduction of the CS1^{bright} population was observed when patient peripheral blood samples were treated with allogeneic PBMCs at an E:T ratio of 10:1 in the presence of the CS1-NKG2D biAb (Fig. 5B). We found that IFNγ production was significantly increased when CS1-NKG2D biAb was added to the coculture containing IL2-primed PBMCs and the CS1^{bright} MM.1.S cell line (Fig. 5C). IL2 priming was observed to be required for this significant induction of IFNγ production (Supplementary Fig. S3). No significant increase in IFNγ production was observed in cocultures of primed PBMCs and CS1^{low} RPMI-8226 cells in the presence of CS1-NKG2D biAb.

The expression of CS1 on normal T, NKT, and NK cells was low and similar to that of the RPMI-8226 MM cell line (Supplementary Fig. S4A). Using autologous PBMCs as target cells, we found no significant increase in specific lysis against autologous T, NKT, or NK cells when cocultured with the CS1-NKG2D biAb compared with the negative control biAb (Supplementary Fig. S4B). We also found that neither soluble CS1 nor an NKG2D binding chimera protein, mimicking the circulating CS1 antigen (28) and shed NKG2D ligands in the peripheral blood of MM patients (29), respectively, affected the CS1-NKG2D biAb-induced cytotoxicity against MM1.S (Supplementary Fig. S5).

**CS1-NKG2D biAb cumulatively induces specific cytolysis of MM**

When IL2-activated PBMCs were used as effectors cells in coculture with the CS1^{high} MM.1.S MM cell line, a significant increase in specific lysis was observed in the presence of CS1-NKG2D biAb compared with the control biAb (Fig. 6A; dashed bracket). When the same experiment was conducted with either CD3-depleted PBMCs (Fig. 6A; green bracket) or CD56-depleted PBMCs (Fig. 6A; red bracket), no significant increase in cytotoxicity was observed in the presence of CS1-NKG2D biAb compared with the same conditions with the control biAb. This suggests that T cells, as well as NK cells, each have a significant role in mediating the enhanced cytotoxic effect noted with total PBMCs plus the CS1-NKG2D biAb. When the cytotoxicity of total PBMCs plus the CS1-NKG2D biAb against the CS1^{high} MM.1.S MM cell line was compared with the same experiment using PBMCs depleted of T cells plus the CS1-NKG2D biAb against the CS1^{high} MM.1.S MM cell line, no significant difference compared with total PBMCs was seen, likely because at the same 10:1 cytotoxicity ratio, the absolute number of NK cells in the coculture with the CS1^{high} MM.1.S MM cell line was increased by approximately 100%. When the same experiment was performed using PBMCs depleted of NK cells plus the CS1-NKG2D biAb against the CS1^{high} MM.1.S MM cell line, significantly lower cytotoxicity compared with total PBMC was observed (Fig. 6A), demonstrating the critical role this minor population of cytolytic effector cells plays in this process. To validate these results, we also used purified CD3^{+} T cells and NK cells as effectors in cocultures with MM1.S cells in the presence of control biAb or the CS1-NKG2D biAb. Similarly, we found that the CS1-NKG2D biAb, but not control biAb, induced a significant increase in cytotoxicity against the MM1.S target cells (Fig. 6B).

To further confirm that the cytotoxicity was from the engagement of the NKG2D receptor, we pretreated the effector PBMC with an anti–NKG2D-blocking antibody prior to the addition of the CS1-NKG2D biAb, and this significantly blunted the enhanced lysis resulting from the addition of the CS1-NKG2D biAb (Fig. 6C). IFNγ production was significantly increased when IL2-primed PBMCs were used as effector cells in coculture with the CS1^{high} MM.1.S MM cells and the CS1-NKG2D biAb compared with the negative control biAb. A profound decrease in IFNγ production was observed when the same experiment was conducted with PBMCs depleted of NKG2D^{+} cells (Fig. 6D). Finally, we used an acute myeloid leukemia (AML) cell line, MOLT-3, as a negative target cell control because it does not express CS1. A significant increase in specific lysis was observed in the presence of the CS1-NKG2D biAb when the CS1 antigen was ectopically expressed on the MOLT-3 AML cell line (Fig. 6E).
CS1-NKG2D biAb treatment prolongs survival of mice bearing human CS1\textsuperscript{high} MM

To determine the efficacy of the CS1-NKG2D biAb in vivo, we used NSG mice engrafted with the MM.1S (CS1\textsuperscript{high}) or NCI-H929 (CS1\textsuperscript{int}) MM cell line. Because the CS1-NKG2D biAb is small, approximately 56 kDa, we first elucidated the optimal protocol in dosing the biAb in vivo. We established an in-house ELISA for quantifying the amount of CS1-NKG2D biAb in mouse plasma, as shown in Supplementary Fig. S6A. Based on the calculated standard curve (Supplementary Fig. S6B), we determined that subcutaneous administration resulted in the longest half-life in vivo when compared with intravenous and intraperitoneal administration (Supplementary Fig. S6C). Based on these data, we injected the mice subcutaneously with 200 μg/kg of CS1-NKG2D biAb or the control biAb twice a day for 2 weeks. For NSG mice engrafted with human PBMCs and CS1\textsuperscript{high} MM.1S MM cells and then treated with the CS1-NKG2D biAb, a significant prolongation of survival over mice engrafted with CS1\textsuperscript{high} MM.1S MM cells alone (P < 0.0001) or with PBMCs and CS1\textsuperscript{high} MM.1S MM cells and then treated with the control biAb (P < 0.0022; Fig. 7). By comparing groups receiving MM.1S MM cells and PBMCs, with or without CS1-NKG2D biAb, a prolonged survival in the group that received CS1-NKG2D biAb (P < 0.01) was also seen. In contrast, there was no difference in survival between the NSG mice engrafted with human PBMCs and H929 MM cells, which have intermediate CS1 expression (Fig. 1E), and NSG mice engrafted with human PBMCs and CS1\textsuperscript{high} MM.1S MM cells that received the negative control biAb. When NSG mice were engrafted with human PBMCs and H929 MM cells, which have intermediate CS1 expression (Fig. 1E), a moderate prolongation of survival in mice that received the CS1-NKG2D biAb was observed, but statistical significance was reached only when compared with mice receiving PBS (P = 0.04, Supplementary Fig. S7). This suggested that the prolonged mouse survival in this experimental MM model was dependent upon the presence of PBMCs, CS1 expression, as well as CS1-NKG2D biAb treatment.

Discussion

The use of biAb or bispecific T/NK cell engager (BiTE or BiKE) constructs is an attractive approach to treat cancer, as it not only simultaneously targets immune cells and tumor-associated antigens, it does so in a fashion that brings these two cell types into close proximity allowing for engagement. Here, we described the construction and functional assessment of a non–IgG-like,
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Figure 6.
Assessment of cytotoxicity and IFNγ production in the presence of the CS1-NKG2D biAb. A, MM1S cell line was cocultured with IL2-primed PBMCs, T cell-depleted PBMCs, or NK cell-depleted PBMCs at an E:T ratio of 10:1 for 24 hours in the presence of biAb. All depletions were from negative depletions. Specific lysis induced from the control biAb and CS1-NKG2D biAb on each effector was compared as shown in dashed (PBMCs), green (T cells), and red (NK cells). B, IL2-primed PBMCs, purified T cells, and purified NK cells were cocultured with the MM1S MM cell line for 24 hours in the presence of biAb. CD3⁺ T cells and CD56⁺ CD3⁻ NK cells were sorted. E:T ratio for NK cells: 0.5:1; E:T ratio for PBMCs and T cells: 10:1. C, Effect of an NKG2D-blocking antibody on the enhanced cytotoxicity of IL2-primed PBMCs induced by the CS1-NKG2D biAb against the MM1S MM cell line at an E:T ratio of 10:1 for 24 hours. The effectors were pretreated with the blockade before coculturing the targets. D, IFNγ production of the PBMCs and NKG2D-depleted PBMCs against the MM1S MM cell line in the presence of biAbs. The E:T ratio is 1:1. E, Cytotoxicity assay of the IL2-primed PBMCs against CS1⁺ MOLT-3 cell line and CS1 overexpressed MOLT-3 cell line. The dose of biAbs for all experiments was 50 μg/mL. Results show specific lysis mean ± SEM of five independent experiments. NS, not significant; *, P < 0.05; **, P < 0.01; ****, P < 0.001 using Student t test for two-group comparison and linear mixed model for multiple group comparison.

scFv-based biAb targeting CS1⁺ MM cells while activating a variety of innate and antigen-specific NKG2D⁺ immune effector cells. We provided evidence to suggest that collective activation of NKG2D⁺ immune cells could enhance cytotoxicity promoted by CD3⁺CD8⁺ T cells or NK cells alone. This CS1-NKG2D biAb is distinct from other reported biAbs or BiTEs using an NKG2D mechanism (30, 31) in that both redirecting subunits are scFvs, and the anti-NKG2D component not only redirects the molecule, but also activates its cognate immune effector cell once fully engaged with its tumor cell target. For human MM cell lines with high surface density expression of CS1, we were able to show that the addition of the CS1-NKG2D biAb, along with the MM target cell and activated human PMBCs, enhanced: (i) the immune synapse between MM target cells and NKG2D⁺ T and NK effector cells; (ii) the phosphorylation of AKT, the downstream signaling molecules of the NKG2D–DAP10 complex (9, 27, 32) within 15 minutes of engagement; (iii) the in vitro cytotoxicity and IFNγ production of NKG2D⁺ effector cells upon exposure to CS1⁺ MM cells; and (iv) the in vivo survival of mice bearing these cell populations, when compared with a negative control biAb. To our knowledge, each of the currently available biAbs targets MM function by redirecting T cells to the tumor cell via CD3. The ULBP2-BB4 bispecific protein construct described by Von Strandmann and colleagues fused an anti-CD138 scFv targeting this MM-associated antigen with ULBP2, a ligand of NKG2D (31). This bispecific protein activated NK cells, and when exposed to CD138⁺ MM cells, induced strong cytotoxicity and IFNγ production in vitro and in vivo. These data, along with our study here, suggest that activation of NKG2D provides a powerful means to eradicate MM cells. Importantly, NKG2D ligands such as MICA/B and ULBP1/2 have an affinity Kᵣ that is in the micromolar range (10). Here, we have shown that the
anti-NKG2D component of the C1-NKG2D biAb had a binding affinity in the nanomolar range, thus potentially leading to a more potent activation of NKG2D+ cytolytic effector cells (21). The possibility exists that C1-NKG2D–activated effector cells could have bystander killing effects on the C1− MM cells. However, in our experiment using MM patient samples as target cells, we found a reduction of the C1+ CD38dim/− CD138+ subset, but not of the C1+ CD38dim− subset. More experiments are needed to confirm the possible bystander effects from the C1-NKG2D–activated effector cells.

The ligands for NKG2D are often expressed by tumor cells, both as membrane bound and in secreted forms, with the latter from the C1-NKG2D biAb. This scFv as membrane bound and in secreted forms, with the latter from the C1-NKG2D biAb. A total of $5 \times 10^6$ MM.1S MM cells and $5 \times 10^7$ PBMCs were used. Dose of control biAb or C1-NKG2D biAb was 200 μg/kg. Data from experiments using human PBMCs of two healthy donors. *P < 0.02, **P < 0.01, ***P < 0.001 using log-rank test.

Figure 7. Prolonged survival in MM bearing NSG mice received human effector cells and C1-NKG2D biAb. A, Kaplan–Meier curve for NSG mice that received MM.1S MM cells alone (n = 4), MM.1S MM cells + human PBMCs + PBS (n = 5), MM.1S MM cells + PBMCs + control biAb (n = 5), or MM.1S MM cells + PBMCs + C1-NKG2D biAb (n = 5). Total of $5 \times 10^6$ MM.1S MM cells and $5 \times 10^7$ PBMCs were used. Dose of control biAb or C1-NKG2D biAb was 200 μg/kg.

Disclosure of Potential Conflicts of Interest

W.K. Chan, M.A. Caligiuri, and J. Yu have submitted a patent through The Ohio State University for the C1-NKG2D bispecific antibody described in this report. No potential conflicts of interest were disclosed by the other authors.

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A CS1-NKG2D Bispecific Antibody CollectivelyActivates Cytolytic Immune Cells against Multiple Myeloma

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