B-cell Maturation Antigen Is a Promising Target for Adoptive T-cell Therapy of Multiple Myeloma

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Abstract

Purpose: Multiple myeloma is a usually incurable malignancy of plasma cells. New therapies are urgently needed for multiple myeloma. Adoptive transfer of chimeric antigen receptor (CAR)–expressing T cells is a promising new therapy for hematologic malignancies, but an ideal target antigen for CAR-expressing T-cell therapies for multiple myeloma has not been identified. B-cell maturation antigen (BCMA) is a protein that has been reported to be selectively expressed by B-lineage cells including multiple myeloma cells. Our goal was to determine if BCMA is a suitable target for CAR-expressing T cells.

Experimental Design: We conducted an assessment of BCMA expression in normal human tissues and multiple myeloma cells by flow cytometry, quantitative PCR, and immunohistochemistry. We designed and tested novel anti-BCMA CARs.

Results: BCMA had a restricted RNA expression pattern. Except for expression in plasma cells, BCMA protein was not detected in normal human tissues. BCMA was not detected on primary human CD34⁺ hematopoietic cells. We detected uniform BCMA cell-surface expression on primary multiple myeloma cells from five of five patients. We designed the first anti-BCMA CARs to be reported and we transduced T cells with lentiviral vectors encoding these CARs. The CARs gave T cells the ability to specifically recognize BCMA. The anti-BCMA-CAR–transduced T cells exhibited BCMA-specific functions including cytokine production, proliferation, cytotoxicity, and in vivo tumor eradication. Importantly, anti-BCMA-CAR–transduced T cells recognized and killed primary multiple myeloma cells.

Conclusions: BCMA is a suitable target for CAR-expressing T cells, and adoptive transfer of anti-BCMA-CAR–expressing T cells is a promising new strategy for treating multiple myeloma.

Introduction

Multiple myeloma is a malignancy characterized by an accumulation of clonal plasma cells (1–3). Current therapies for multiple myeloma often cause remissions, but nearly all patients eventually relapse and die (1, 2). There is substantial evidence of an immune-mediated elimination of myeloma cells in the setting of allogeneic hematopoietic stem cell transplantation; however, the toxicity of this approach is high, and few patients are cured (1, 4). Although some monoclonal antibodies have shown promise for treating multiple myeloma in preclinical studies and early clinical trials, consistent clinical efficacy of any monoclonal antibody therapy for multiple myeloma has not been conclusively shown (5–7). There is clearly a great need for new immunotherapies for multiple myeloma, and developing an effective antigen-specific adoptive T-cell therapy for this disease would be a major advance.

Adoptive transfer of T cells genetically modified to recognize malignancy-associated antigens is a promising approach for cancer therapy (8, 9). T cells can be genetically modified to express chimeric antigen receptors (CAR), which are fusion proteins that include an antigen recognition moiety and T-cell activation domains (9, 10). For B-lineage malignancies, substantial progress has been made recently in developing adoptive T-cell approaches that use anti-CD19 CARs (11–18). Anti-CD19-CAR–transduced T cells have cured leukemia and lymphoma in mice (19, 20). Several patients obtained remissions in early clinical trials of adoptively transferred anti-CD19-CAR–transduced T cells, and T cells transduced with anti-CD19 CARs also eradicated normal B cells (12, 13, 17, 21). Unfortunately, CD19 is rarely expressed in the malignant plasma cells of multiple myeloma, so treating multiple myeloma with CAR-expressing T cells will require identifying other antigens to target (22, 23).
Anti-BCMA Chimeric Antigen Receptor

Translational Relevance

One way to improve outcomes of patients with multiple myeloma might be to develop effective immunotherapies targeting antigens expressed by multiple myeloma cells. T cells expressing chimeric antigen receptors (CAR) that target the B-cell antigen CD19 have potent in vivo activity. Unfortunately, a suitable target for CAR-expressing T-cell therapy for multiple myeloma has not been previously identified because most proteins expressed in multiple myeloma cells are also expressed in essential normal cells. We assessed B-cell maturation antigen (BCMA) as a possible target for CAR-expressing T cells. Our results show that BCMA is expressed uniformly on the malignant plasma cells of many patients with multiple myeloma. BCMA expression was not detected on essential normal cells. We designed the first anti-BCMA CARs to be reported. T cells expressing these CARs could recognize and destroy primary human multiple myeloma cells. These findings are the first steps toward clinical trials of anti-BCMA CAR-expressing T cells.

One candidate antigen of immunotherapies for multiple myeloma is B-cell maturation antigen (BCMA, CD269; refs. 24, 25). BCMA RNA was detected universally in multiple myeloma cells, and BCMA protein was detected on the surface of plasma cells from patients with multiple myeloma by several investigators (26–29). BCMA is a member of the TNF receptor superfamily (30, 31). BCMA binds B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL; refs. 31–33). Among nonmalignant cells, BCMA has been reported to be expressed mostly by plasma cells and subsets of mature B cells (24, 25, 32, 34, 35). Mice deficient in BCMA were healthy and had a normal physical appearance (36, 37). BCMA-deficient mice had normal numbers of B cells, but survival of long-lived plasma cells was impaired (34, 36).

We reasoned that BCMA would be an appropriate target antigen for treating multiple myeloma with CAR-expressing T cells. Except for expression in plasma cells, we found that BCMA is not expressed in the cells of major human organs. We designed lentiviral vectors that encoded BCMA-specific CARs. T cells transduced with these vectors performed BCMA-specific functions including cytokine production, proliferation, and cytotoxicity.

Materials and Methods

Cell lines and primary cells

H929, U266, and RPMI-8226 are all BCMA+ multiple myeloma cell lines that were obtained from American Type Culture Collection (ATCC). A549 is a BCMA-negative lung cancer cell line (ATCC). TC71 is a BCMA-negative sarcoma cell line. CCRF-CEM is a BCMA-negative T-cell line (ATCC). BCMA-K562 are K562 cells (ATCC) transduced with the gene for full-length BCMA in our laboratory. NGFR-K562 are K562 cells transduced with the gene for low-affinity nerve growth factor in our laboratory (38). The same gammaretroviral vector and methods were used to transduce BCMA-K562 and NGFR-K562. We used tissue samples or peripheral blood mononuclear cells (PBMC) from 6 patients with multiple myeloma designated myeloma patients 1 to 6. We used PBMC from 3 subjects with melanoma: donor A, B, and C. We obtained primary CD34+ hematopoietic cells from 3 healthy normal donors. All of the human samples mentioned earlier were obtained from patients enrolled on Institutional Review Board (IRB)-approved clinical trials at the National Cancer Institute (Bethesda, MD).

Real-time qPCR to quantify BCMA transcript copies

We quantitated BCMA cDNA copies in samples of cDNA from human tissues included in the Human Major Tissue qPCR panel II (Origene) by conducting quantitative PCR (qPCR) with a BCMA-specific primer and probe set (Applied Biosystems, catalog number 4331182). As a positive control, we quantitated BCMA cDNA copies in cDNA of multiple myeloma cells from a plasmacytoma of a patient with advanced multiple myeloma. RNA was extracted from the plasmacytoma cells with an RNeasy mini kit (Qiagen), and cDNA was synthesized with standard methods. A standard curve for the BCMA qPCR was created by amplifying dilutions of a plasmid that encoded the full-length cDNA of BCMA (Origene). The qPCR accurately detected copy numbers from 102 to 109 copies of BCMA per reaction. We also quantitated the number of β-actin cDNA copies in the same tissues with a TaqMan β-actin primer and probe kit (Applied Biosystems). A β-actin standard curve was created by amplifying serial dilutions of a β-actin plasmid. All qPCR reactions were carried out on a Roche LightCycler480 machine.

Immunohistochemistry

BCMA stains were conducted on formalin-fixed paraffin-embedded tissue sections and on a commercial normal human tissue array (Pantomics Inc., catalog number MN0661). NGFR-K562 cells (BCMA-negative), BCMA-K562 cells (BCMA-positive), and sections from reactive tonsils were used as controls. Sections were deparaffinized in xylene, rehydrated in graded-alcohol, placed in 1× low pH antigen retrieval solution (Dako, catalog number S1699), and steamed for 30 minutes. Sections were blocked with Dako protein block serum-free (catalog number X0909) for 30 minutes, and blocked with 5% bovine serum albumin (Sigma-Aldrich) for another 30 minutes. The sections were then incubated for 60 minutes at room temperature with either 1 µg/mL anti-BCMA goat polyclonal antibody (R&D Systems, catalog number AF193) or with an isotype control of 1 µg/mL normal purified goat immunoglobulin G (IgG). Biotinylated rabbit anti-goat IgG was used as the secondary antibody (Vector Lab, catalog number BA-5000; 7.5 µg/mL, 30-minute incubation, room temperature). Vectastain ABC Kit (Vector Lab, catalog number...
PK4005; 30-minute incubation, room temperature) and DAB-+Chromogen (Dako, catalog number K4011; 5-minute incubation, room temperature) were used for detection. For BCMA and multiple myeloma 1 (MUM1) double staining, BCMA stained sections were re-retrieved in hot Dako 1× low pH antigen retrieval solution and microwaved for 6 minutes. Sections were blocked with Dako protein block serum-free for 30 minutes, incubated with MUM1 mouse monoclonal antibody (Dako, M7259; 1:100 dilution, 120-minute incubation, room temperature) and detected with Ventana BenchMark XT with Ventana ultraView universal alkaline phosphatase red detection kit (AZ, A760-501). Images were taken with an Olympus Bx41 microscope: objective UPPlanFL 100×/1.30 oil, adaptor U-TV0.5×C, digital camera Q-imaging Micropublisher 5.0 RTV. The images were captured with "Q-Capture Version 3.1" and imported to Adobe Photoshop 7.0.

Construction of anti-BCMA CARs

Sequences of 2 mouse anti-human BCMA antibodies were obtained from a patent (39). The specific antibodies used were designated in the patent as C12A3.2 and C11D5.3. The heavy chain and light chain variable-region sequences of these antibodies were used to design single chain variable fragments (scFv) with the following pattern: light chain variable region-linker-heavy chain variable region. The linker had the following amino acid sequence: GSTSGSGKPGSGEGSTKG (15). CARs incorporating variable regions from C12A3.2 and C11D5.3 were designated anti-bcma1 and anti-bcma2, respectively. DNA sequences encoding these CARs were designed. The sequence of each CAR followed this pattern from the 5’-end to the 3’-end: the CD8α signal sequence, scFv, hinge and transmembrane regions of the human CD8α molecule, the cytoplasmic portion of the CD28 molecule, and the cytoplasmic portion of the CD3ζ molecule. The sequences used for CD8α, CD28, and CD3ζ were obtained from the National Center for Biotechnology Information (Bethesda, MD) website (www.ncbi.nlm.nih.gov/). Guidance about the portions of each molecule to include in the CARs was obtained from prior work (38). DNA encoding the CARs was codon-optimized and synthesized by GeneArt AG with appropriate restriction sites. The CAR sequences were ligated into a lentiviral vector plasmid designated pRRLSIN.cPPT.MSCV.anti-bcma1.oPRE (40). The coDMF5 portion of this vector was replaced with the CAR sequences by using standard methods. Two anti-BCMA–encoding CAR vectors were constructed: pRRLSIN.cPPT.MSCV.anti-bcma1.oPRE and pRRLSIN.cPPT.MSCV.anti-bcma2.oPRE. A negative-control CAR that contained the SP6 scFv, which recognized the hapten 2,4,6-trinitrophenyl, was also constructed (10). This CAR was referred to as SP6. The SP6 CAR was cloned into the same lentiviral vector as the anti-BCMA CARs and contained the same signaling domains as the anti-BCMA CARs.

Lentiviral supernatant production

Supernatant that contained lentiviruses encoding each CAR was produced by following a previously published protocol (40). To produce the supernatant, 293T-17 cells (ATCC) were transduced with the following plasmids as detailed previously: pMDG (encoding the vesicular stomatitis virus envelope), pMDLg/pRRE (encoding gag and pol), pRSV-Rev (encoding Rev), and the appropriate CAR-encoding plasmid (40).

T-cell transductions

T cells were cultured as described previously (38). In brief, PBMC were stimulated with the anti-CD3 monoclonal antibody OKT3 (Ortho) in AIM V medium (Invitrogen) containing 5% human blood type AB serum (Valley Biomedical) and 300 international units (IU)/mL of interleukin-2 (IL-2; Chiron). Thirty-six hours after the cultures were started, the activated PBMC were suspended in lentiviral supernatant with protamine sulfate and 300 IU/mL IL-2. The cells were centrifuged for 1 hour at 1,200 × g. The cells were then cultured for 3 hours at 37°C. Next, the supernatant was diluted 1:1 with RPMI (Mediatech)+10% FBS (Invitrogen) and IL-2. The cells were cultured in the diluted supernatant overnight and then they were returned to culture in AIM V medium plus 5% human AB serum with IL-2.

CAR detection on transduced T cells by anti-Fab antibody staining

T cells were stained with biotin-labeled polyclonal goat anti-mouse-F(ab)2 antibodies (anti-Fab; Jackson Immunoresearch) to detect the anti-BCMA CARs as described previously (38).

Flow cytometry

For anti-BCMA staining, cells were stained with polyclonal biotin-labeled goat anti-human BCMA antibodies (R&D, catalog number BAF 193) followed by streptavidin (BD Biosciences). Bone marrow cells were also stained with anti-CD38 (eBioscience) and anti-CD56 (BD Biosciences). Flow cytometry analysis for all experiments was carried out by using FlowJo (Tree Star, Inc.).

ELISA, CD107a, intracellular cytokine staining, and proliferation assays

These assays were conducted by using standard methods, as previously published (17, 38). Details of the methods for these techniques are in the Supplementary Methods.

Cytotoxicity assay

Cytotoxicity assays were conducted as previously described (38). Cytotoxicity was measured by comparing survival of BCMA+ target cells relative to the survival of negative-control CCRF-CEM cells. Both of these cell types were combined in the same tubes with CAR-transduced T cells. CCRF-CEM-negative control cells were labeled with the fluorescent dye 5-(and-6)-((4-chloromethyl)benzoyl)amino) tetracyramyethylrhodamine (CM1MR; Invitrogen), and BCMA+ target cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). Cocultures were set up in sterile 5 mL test tubes (BD Biosciences) in duplicate at multiple T cell to target cell ratios. The target cells contained in the tubes were 50,000...
BCMA+ target cells along with 50,000 CCRF-CEM–negative control cells. The cultures were incubated for 4 hours at 37°C. Immediately after the incubation, 7-AAD (7-amino-actinomycin D; BD Biosciences) was added and flow cytometry acquisition was conducted. For each T cell plus target-cell culture, the percentage survival of BCMA+ target cells was determined by dividing the percentage live BCMA+ cells by the percentage live CCRF-CEM–negative control cells. The corrected percentage survival of BCMA+ target cells was calculated by dividing the percentage survival of BCMA+ target cells in each T cell plus target cell culture by the ratio of the percentage live BCMA+ target cells to percentage live CCRF-CEM–negative control cells in tubes containing only BCMA+ target cells and CCRF-CERM cells without effector T cells. This correction was necessary to account for variation in the starting cell numbers and for spontaneous target cell death. Cytotoxicity was calculated as follows: the percentage cytotoxicity of BCMA+ target cells = 100 \times \frac{\text{percentage survival of BCMA+ target cells}}{\text{percentage survival of BCMA– target cells}}.

In vivo treatment model murine experiments

NSG mice (NOD.Cg-Prkdcscid Il2rgtm1WjlSzJ) from The Jackson Laboratory were used. Mice received intradermal injections of RPMI-8226 cells. Tumors were allowed to grow for 17 to 19 days, and then the mice received intravenous infusions of 8 \times 10^6 human T cells that were transduced with either anti-bcma2 or SP6. Tumors were measured with calipers every 3 days. The longest length and the length perpendicular to the longest length were multiplied to obtain the tumor size (area) in mm^2. When the longest length reached 15 mm, mice were sacrificed. Animal studies were approved by the National Cancer Institute Animal Care and Use Committee.

Results

BCMA was expressed in multiple myeloma cell lines but not in several other types of cell lines

We used flow cytometry to characterize the expression of BCMA on the cell surfaces of a variety of cells (Fig. 1A). As expected, K562 cells transduced with the gene for BCMA expressed cell-surface BCMA. The multiple myeloma cell lines H929, U266, and RPMI-8226 also expressed cell-surface BCMA. In contrast, NGFR-K562 cells, the sarcoma cell line TC71, the T-cell leukemia line CCRF-CERM, and the kidney cell line 293T-17 did not express cell-surface BCMA. Importantly, primary CD34+ hematopoietic cells lacked cell-surface BCMA expression.

BCMA had a restricted mRNA expression pattern

A critical factor for any antigen being considered as a target for immunotherapies is the antigen’s expression pattern in normal tissues. BCMA has been reported to be expressed on plasma cells and on some B cells but to otherwise have limited expression (24–26, 35). To more completely assess the expression pattern of BCMA, we conducted qPCR on a panel of cDNA samples from a wide range of normal tissues (Fig. 1B). As a positive control, we conducted qPCR on cDNA from cells of a plasmacytoma that was resected from myeloma patient 1, a patient with advanced multiple myeloma. Ninety-three percent of the cells from the plasmacytoma sample were plasma cells as determined by flow cytometry. The BCMA expression of the plasmacytoma sample was dramatically higher than the BCMA expression of any other tissue. Not surprisingly, BCMA cDNA copies were detected in several hematologic tissues, such as blood leukocytes, bone marrow, spleen, lymph node, and tonsil. Low levels of BCMA cDNA copies were detected in the samples of testis and trachea. In addition, low levels of BCMA cDNA copies were detected in most gastrointestinal organs, such as duodenum, rectum, and stomach. One possible explanation for BCMA expression in gastrointestinal organs and the trachea was the known presence of plasma cells and B cells in tissues such as lamina propria and Peyer’s Patches (41, 42).

BCMA protein was expressed by multiple myeloma cells. Except for plasma cells, BCMA protein expression was not detected in normal human organs

After showing a very restricted expression pattern of BCMA RNA, we carried out an assessment of BCMA protein expression by immunohistochemistry (IHC). As expected, our anti-BCMA immunohistochemical staining procedure yielded strong staining of BCMA-K562 cells and a lack of staining with NGFR-K562–negative control cells (Fig. 2A and B). We went on to evaluate BCMA protein expression in normal human organs. Except for plasma cells, we did not detect BCMA protein expression by the cells of any of the organs that we stained. We detected plasma cells expressing cell-surface BCMA in gastrointestinal organs. Examples of BCMA-expressing plasma cells that were detected in the stomach and rectum are shown in Fig. 2C and D. MUM1 is a protein that is expressed by many malignant and benign lymphocytes and by plasma cells (43). Except for lymphocytes and plasma cells, MUM1 is not expressed by normal gastrointestinal tissues (43). We conducted immunohistochemical double staining of colon and duodenum with anti-BCMA and anti-MUM1 and found that BCMA was expressed only by lymphoid cells and plasma cells (Fig. 2E). BCMA expression by plasma cells probably accounts for the low levels of BCMA RNA detected in these organs because we did not detect BCMA expression by any of the other cells in these organs. We detected BCMA-expressing plasma cells in the tonsil (data not shown). The organs assessed by IHC and found to lack BCMA expression except for plasma cells included the following: adrenal, bladder, bone, eye, breast, cerebellum, cerebral cortex, fallopian tube, esophagus, stomach, small intestine, colon, rectum, heart, kidney, liver, lung, ovary, pancreas, parathyroid, pituitary, placenta, prostate, skin, spinal cord, spleen, skeletal muscle, testis, thymus, thyroid, trachea, cervix, and uterine endometrium.

For a protein to be an appropriate target for CAR-expressing T cells aimed at multiple myeloma, the protein must be expressed on the surface of multiple myeloma cells. To assess BCMA expression by multiple myeloma cells, we
Figure 1. BCMA had a restricted pattern of expression. A, cell-surface BCMA was detected on multiple myeloma cell lines, but BCMA was not detected in other cell lines. For all plots, the solid line represents staining with anti-BCMA antibodies, and the dashed line represents staining with isotype-matched control antibodies. BCMA was expressed by BCMA-K562 cells but not by NGFR-K562 cells. Flow cytometry staining revealed BCMA on the cell surface of the multiple myeloma cell lines H929, U266, and RPMI-8226. BCMA was not detected in the sarcoma cell line TC71, in the T cell leukemia line CCRF-CEM, or in the kidney cell line 293T-17. Primary CD34⁺ hematopoietic cells lacked cell-surface BCMA expression. All plots are gated on live cells. The primary CD34⁺ cells plot is also gated on CD34⁺ cells.

B, BCMA cDNA copies were measured in samples of the indicated normal tissues by qPCR. In addition, qPCR was conducted on cDNA from cells of a plasmacytoma from myeloma patient 1 as a positive control. Neoplastic plasma cells made up 93% of the total cells in the sample. Actin copies were also measured by qPCR in all of the samples, and the results were expressed as the number of BCMA cDNA copies per 10⁵ actin cDNA copies.
stained tissue sections from 3 different patients with multiple myeloma (Fig. 2F–H). These sections are shown in Fig. 2. In all 3 of the samples, the neoplastic plasma cells expressed cell-surface BCMA.

BCMA-specific CARs were expressed on transduced T cells

BCMA is expressed in multiple myeloma cells, and it has a restricted expression pattern in normal tissues (Figs. 1 and 2). Because of the expression pattern of BCMA, we reasoned that BCMA would be an appropriate target for CAR-expressing T cells. To further assess the suitability of BCMA as a target for CAR-expressing T cells, we designed 2 CARs. Each CAR contained an scFv derived from 1 of 2 mouse anti-human BCMA monoclonal antibodies (39). We named the 2 CARs anti-bcma1 and anti-bcma2. The CARs contained the hinge and transmembrane regions of the human CD8α molecule, the signaling moiety of the CD28 costimulatory molecule, and the signaling domains of the CD3ζ molecule (Fig. 3A). We also designed a negative control CAR, named SP6. The SP6 CAR contained the variable regions of the hapten-specific SP6 monoclonal antibody (10). Except for the different variable regions, the sequence of the SP6 CAR was identical to the sequences of anti-bcma1 and anti-bcma2. We ligated DNA encoding each of the CARs that we designed into a self-inactivating lentiviral vector, which has been described in detail previously (40). Replication-competent lentiviruses encoding the CARs were used to transduce human T cells. After transductions, we found high levels of cell-surface

![Anti-BCMA Chimeric Antigen Receptor](image_url)
expression of anti-bcma1, anti-bcma2, and SP6 on the transduced T cells (Fig. 3B).

**Anti-BCMA-CAR–transduced T cells degranulated and produced cytokines specifically in response to BCMA-expressing target cells**

Anti-BCMA-CAR–transduced T cells upregulated CD107a specifically in response to stimulation with BCMA-expressing target cells (Fig. 3C). This indicates BCMA-specific degranulation of the T cells, which is a requirement for perforin-mediated cytotoxicity (44).

We assessed the ability of anti-BCMA-CAR–transduced T cells to produce cytokines in response to BCMA. T cells transduced with either anti-bcma1 or anti-bcma2 produced large amounts of IFN-γ when they were cultured overnight with the BCMA-expressing target cell line BCMA-K562, but the CAR-transduced T cells only produced background levels of IFN-γ when they were cultured with the negative-control target cell line NGFR-K562 (Table 1). T cells transduced with anti-BCMA CARs produced large amounts of IFN-γ when they were cultured with a variety of BCMA-negative target cell lines. In contrast, the anti-BCMA CARs produced only background levels of IFN-γ when they were cultured with a variety of BCMA-negative target cell lines. T cells transduced with the SP6 negative control CAR produced only low levels of IFN-γ when cultured with any of the cell lines. The SP6 CAR specifically produced IFN-γ when stimulated with cells coated with its

Figure 3. Anti-BCMA CARs were expressed on the surface of T cells. A, a general diagram of anti-BCMA CAR fusion proteins is shown (LTR, long terminal repeat; MSCV, mouse stem cell virus; SIN, self-inactivating). From the N-terminus to the C-terminus, the anti-BCMA CARs all included an anti-BCMA scFv, the hinge, and transmembrane (TM) regions of the CD8ε molecule, the cytoplasmic portion of the CD28 molecule, and the cytoplasmic portion of the CD3ζ molecule. B, T cells from donor B were transduced with lentiviral vectors encoding 1 of 3 CARs or left untransduced (UT). Expression of 2 anti-BCMA CARs, which were designated anti-bcma1 and anti-bcma2, was detected by flow cytometry 5 days after the transduction. Expression of the control CAR SP6 was also detected. As controls, isotype-matched antibody staining was included for each cell type. The plots are gated on CD3⁺ lymphocytes. The numbers on the plots are the percentages of cells in each quadrant. This is one of 7 experiments with similar results. C, T cells were transduced with either the BCMA-expressing cell line BCMA-K562 or the BCMA-negative cell line NGFR-K562 for 4 hours. After transduction with anti-bcma2, T cells degranulated in a BCMA-specific manner. SP6-transduced T cells from the same donors did not degranulate in a BCMA-specific manner. The plots are gated on live CD3⁺ lymphocytes. The numbers on the plots are the percentages of cells in each quadrant. This is 1 of 3 experiments with similar results.
target, the hapten 2,4,6-trinitrophenyl (data not shown). T cells expressing either anti-bcma1 or anti-bcma2 were functionally similar. Compared with anti-bcma1–transduced T cells, anti-bcma2–transduced T cells consistently exhibited slightly stronger and more specific recognition of BCMA-expressing target cells in ELISA, CD107a, and other functional assays; therefore, we used anti-bcma2 in our remaining experiments.

We successfully cultured and transduced the T cells of myeloma patient 4, a patient who had received 13 prior cycles of therapy for her myeloma. Six days after the cultures were initiated, anti-bcma2 was detected in 69% of the T cells. Large populations of anti-bcma2–transduced T cells from myeloma patient 4 produced IL-2, TNF, and IFN-γ in a BCMA-specific manner after a 6-hour stimulation with BCMA-expressing target cells (Fig. 4A and Supplementary Fig. S1).

### Anti-BCMA-CAR–transduced T cells proliferated in response to BCMA

We assessed anti-bcma2–transduced T cells for the ability to proliferate when stimulated with BCMA-expressing target cells. We cultured CFSE-labeled, anti-bcma2–transduced T cells with either target cells that expressed BCMA or target cells that did not express BCMA (Fig. 4B). Anti-bcma2–transduced T cells specifically proliferated when stimulated with BCMA-expressing target cells. As expected, there was no BCMA-specific proliferation when T cells transduced with the SP6 negative control CAR were assessed. At the beginning of the proliferation assay reported in Fig. 4B, 0.8 × 10^6 anti-bcma2–expressing T cells were cultured with either BCMA-K562 or NGFR-K562 cells. After 4 days of culture, 2.7 × 10^6 anti-bcma2–expressing T cells were present in the cultures containing BCMA-K562 cells, whereas only 0.6 × 10^6 anti-bcma2–expressing T cells were present in the cultures containing NGFR-K562 cells. This BCMA-specific increase in the absolute number of anti-bcma2–expressing T cells confirms the results of the CFSE proliferation assay.

### Anti-BCMA-CAR–transduced T cells killed multiple myeloma cell lines

T cells transduced with anti-bcma2 specifically killed the BCMA-expressing multiple myeloma cell lines H929 and RPMI-8226 in 4-hour cytotoxicity assays (Fig. 4C and D). SP6-transduced T cells exhibited much lower levels of cytotoxicity against these cell lines.

### Anti-BCMA-CAR–transduced T cells eradicate tumors in vivo

We established RPMI-8226 human multiple myeloma cell line tumors in immunodeficient mice. We allowed sizable tumors to develop over 17 to 19 days, and then we treated the mice with a single intravenous infusion of anti-bcma2–transduced human T cells. The anti-bcma2–transduced T cell infusion cured 100% of the mice, with dramatic regressions of all tumors occurring between day 6 and 15 after the T-cell infusion (Fig. 4E and F). In contrast, tumors continued to increase in size in all mice receiving infusions of T cells expressing a negative control CAR-designated SP6. The mice receiving infusions of anti-bcma2–transduced T cells had no signs of toxicity during this experiment.

### Soluble BCMA protein does not interfere with anti-BCMA CAR function

BCMA can be found in the serum of humans and serum BCMA levels are elevated in patients with multiple myeloma (45). Other investigators have previously shown that CAR-expressing T cells were not blocked from recognizing target cells by soluble protein in vitro or in vivo (46). To determine if anti-bcma2 is blocked by soluble BCMA, we conducted ELISA assays in which anti-bcma2–transduced T cells were cultured with BCMA-expressing target cells. Graded concentrations of BCMA protein were added to the culture medium. BCMA protein concentrations of more than 10-fold higher than the median BCMA levels found in the serum of patients with multiple myeloma did not block recognition of BCMA target cells by anti-bcma2–transduced T cells in vitro (Supplementary Fig. S2); furthermore, we

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**Table 1. Anti-BCMA-CAR–transduced T cells specifically recognized BCMA**

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<thead>
<tr>
<th>Effector cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BCMA-expressing targets&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BCMA-negative targets</th>
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<tr>
<td></td>
<td>BCMA-K562</td>
<td>H929</td>
</tr>
<tr>
<td>Anti-bcma1</td>
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<td>Targets alone</td>
<td>&lt;12</td>
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<sup>a</sup>Effector cells were T cells from a patient with multiple myeloma (myeloma patient 4). The T cells were transduced with the indicated CAR or left untransduced.

<sup>b</sup>The indicated target cells were combined with the effector cells for an overnight incubation and an IFN-γ ELISA was conducted. All units are pg/mL IFN-γ.
Figure 4. T cells expressing anti-BCMA CARs produced cytokines, proliferated, and killed in a BCMA-specific manner. Anti-BCMA CAR-transduced T cells eradicated tumors in vivo. A, T cells from myeloma patient 4 were transduced with either anti-bcma2 or the negative-control CAR SP6. Four days later, the CAR-transduced T cells were cultured for 6 hours with either the BCMA-expressing cell line BCMA-K562 or the BCMA-negative cell line NGFR-K562. Large fractions of the T cells transduced with anti-bcma2 produced IL-2 when cultured with BCMA-K562. Only small numbers of anti-bcma2-transduced T cells produced IL-2 when they were cultured with NGFR-K562. Only small numbers of SP6-transduced T cells produced IL-2 when cultured with either BCMA-K562 or NGFR-K562. The plots are gated on CD3+ lymphocytes. The numbers on the plots are the percentages of cells in each quadrant. This is 1 of 3 experiments with similar results. B, T cells from donor B were transduced with either anti-bcma2 or SP6. The T cells were then labeled with CFSE. The T cells were cultured with either irradiated BCMA-K562 cells or irradiated NGFR-K562 cells. IL-2 was not included in the cultures. Four days later, the T cells were analyzed by flow cytometry. The plots are gated on CAR-expressing T cells. The CFSE fluorescence was less intense in anti-bcma2-expressing T cells that were cultured with BCMA-K562 cells (solid) than in anti-bcma2-expressing T cells that were cultured with NGFR-K562 cells (dashed, open). This indicates that anti-bcma2-expressing T cells proliferated specifically in response to BCMA. For SP6-expressing T cells, CFSE fluorescence intensity was similar for T cells cultured with either BCMA-K562 or NGFR-K562. This is 1 of 2 experiments with similar results. Anti-bcma2-transduced T cells from donor A specifically killed the multiple myeloma cell lines (C) H929 and (D) RPMI-8226 in 4-hour cytotoxicity assays at various effector:target cell ratios. T cells transduced with the negative control CAR SP6 caused much lower levels of cytotoxicity at all effector:target ratios. For all effector:target ratios, the cytotoxicity was determined in duplicate, and the results are displayed as the mean ± SEM. E, mice were injected intradermally with RPMI-8226 cells and tumors were allowed to grow for 17 to 19 days. On day 0, the mice received intravenous infusions of 8 × 106 T cells that were transduced with either anti-bcma2 or the negative control CAR SP6. Another group of mice was left untreated. The mice all had established tumors at the time of T-cell infusion. One-hundred percent of mice receiving infusions of anti-bcma2 had rapid and complete regressions of their tumors. In contrast, all mice receiving infusions of SP6-transduced T cells and all mice left untreated had progressive enlargement of their tumors. F, tumors did not recur in mice receiving anti-bcma2-transduced T cells for the duration of the experiment. All mice receiving anti-bcma2-transduced T cells survived and were healthy for the duration of the experiment. All mice receiving SP6-transduced T cells or left untreated died with progressive tumors. The P < 0.0001 refers to the comparison of anti-bcma2 and SP6. E and F, combined results of 3 experiments (anti-bcma2, n = 10; SP6, n = 10; untreated, n = 7).
found that RPMI-8226 cells secreted BCMA, and mice bearing RPMI-8226 tumors had easily detectable serum human BCMA (Supplementary Fig. S3), so serum BCMA did not preclude effective treatment of BCMA + tumors in mice (Fig. 4E and F).

Anti-BCMA-CAR–transduced T cells recognized and killed primary multiple myeloma cells

We detected BCMA expression in neoplastic plasma cells of myeloma patients 1 through 3 by IHC (Fig. 2). We also assessed cell-surface BCMA expression on primary multiple myeloma cells from myeloma patients 1, 5, and 6 by flow cytometry, and we found uniform cell-surface BCMA expression on the plasma cells of all 3 patients. Overall, we found cell-surface BCMA expression on multiple myeloma cells from 5 of 5 unique patients by either IHC or flow cytometry. Examples of flow cytometry staining for BCMA are shown in Fig. 5A and C.

We conducted a series of experiments to show that T cells transduced with anti-bcma2 could specifically recognize primary multiple myeloma cells. Plasma cells that uniformly expressed cell-surface BCMA made up 40% of the cells in a bone marrow sample from myeloma patient 5 (Fig. 5A). Allogeneic anti-bcma2–transduced T cells from donor C produced IFN-γ after coculture with the unmanipulated myeloma-containing bone marrow cells of myeloma patient 5 (Fig. 5B). Anti-bcma2–transduced T cells from the same allogeneic donor produced much less IFN-γ when they were cultured with PBMC from myeloma patient 5. In addition, SP6-transduced T cells from donor C did not specifically recognize the myeloma-containing bone marrow of myeloma patient 5. Other investigators have previously reported that normal PBMC did not contain cells that expressed BCMA (35). We assessed the PBMC of myeloma patient 5 for BCMA expression by flow cytometry. We found that the PBMC did not contain BCMA-expressing cells except for a small population of CD56 + CD38 high cells that made up approximately 0.75% of the PBMC. This population probably consisted of circulating multiple myeloma cells.

Plasma cells made up 93% of the cells from a plasmacytoma resected from myeloma patient 1, and flow cytometry revealed that these primary plasma cells uniformly expressed BCMA (Fig. 5C). BCMA expression was also detected in the neoplastic plasma cells of myeloma patient 1 by qPCR and by IHC (Figs. 1B and 2). Anti-bcma2–transduced T cells from myeloma patient 4 produced IFN-γ when cultured with the allogeneic, unmanipulated multiple myeloma cells of myeloma patient 1 (Fig. 5D). Anti-bcma2–transduced T cells from myeloma patient 4 did not produce significant amounts of IFN-γ when cultured with PBMC from myeloma patient 1. SP6-transduced T cells from myeloma patient 4 did not produce significant amounts of IFN-γ when they were cultured with either multiple myeloma cells or PBMC from myeloma patient 1. The PBMC of myeloma patient 1 did not express BCMA as measured by flow cytometry (data not shown).

We successfully cultured and transduced the T cells of myeloma patient 1, who had received 8 prior cycles of myeloma therapy. Eight days after the cultures were initiated, anti-bcma2 was detected in 65% of the T cells. The anti-bcma2–transduced T cells from myeloma patient 1 produced IFN-γ specifically in response to autologous multiple myeloma cells (Fig. 5E). SP6-transduced T cells from myeloma patient 1 did not recognize autologous multiple myeloma cells. Neither anti-bcma2–transduced T cells nor SP6-transduced T cells from myeloma patient 1 recognized autologous PBMC. Anti-bcma2–transduced T cells from myeloma patient 1 specifically killed autologous multiple myeloma cells at low effector to target ratios; in contrast, SP6-transduced T cells from myeloma patient 1 exhibited low levels of cytotoxicity against autologous multiple myeloma cells (Fig. 5F).

Discussion

Because current therapies for multiple myeloma are rarely curative, new therapies for this disease are clearly needed (1, 2). In particular, there is a need for therapies to eradicate residual malignant cells that persist in patients who obtain remissions with current treatments. Many monoclonal antibodies that could potentially be useful therapies for multiple myeloma are currently being evaluated in clinical trials, but most of these antibodies target antigens that are expressed in both essential normal cells and myeloma cells (5, 7). Severe toxicities have occurred after infusions of genetically modified T cells targeting antigens expressed by normal epithelial tissues; therefore, when designing CAR-expressing T-cell therapies, it is prudent to avoid targeting antigens that are expressed on the cell surfaces of normal epithelial cells (47, 48). Identification of new antigens that are expressed by multiple myeloma cells but not by normal essential cells is a critical step for development of effective immunotherapies for multiple myeloma.

We chose to investigate the TNF-receptor superfamily member BCMA as a possible target for CAR-expressing T cells. CAR-expressing T cells recognize cell-surface antigens (9); therefore, an antigen targeted by CAR-expressing T cells should ideally be expressed on the cell surfaces of malignant cells but not on the cell surfaces of essential normal cells. BCMA has been previously detected on the cell surfaces of multiple myeloma cells by other investigators (26–28). In early studies, BCMA RNA was detected in a limited number of normal tissues (24, 25). BCMA RNA was not expressed by normal human T cells or myeloid cells (35). BCMA has been reported to be expressed by fibroblast-like synovial cells from patients with the autoimmune disease rheumatoid arthritis, but BCMA was not expressed by synovial cells from patients with osteoarthritis (49). BCMA mRNA has been previously detected in the gut-associated lymphoid tissues, which are known to contain B cells and plasma cells (41, 50). We measured BCMA transcript expression in a wide range of normal tissues and found that BCMA mRNA expression was quite limited (Fig. 1B). We also determined that BCMA was not expressed on the cell surfaces of primary CD34 + hematopoietic cells (Fig. 1A). Finally, we conducted an immunohistochemical analysis of BCMA protein...
Figure 5. Anti-BCMA-CAR–transduced T cells specifically recognized and killed BCMA-expressing primary multiple myeloma (MM) cells. A, flow cytometry staining for BCMA (solid line) and isotype-matched control staining (dashed line) revealed BCMA expression on the surface of primary bone marrow multiple myeloma cells from myeloma patient 5. The plot is gated on CD38<sup>high</sup> CD56<sup>+</sup> plasma cells, which made up 40% of the bone marrow cells. B, unmanipulated myeloma-containing bone marrow cells (MM) from myeloma patient 5 or PBMC from myeloma patient 5 were cocultured overnight with allogeneic T cells from donor C. The T cells had been transduced with either anti-bcma2 or the negative control CAR SP6. After the coculture, an IFN-γ ELISA was conducted. C, flow cytometry for BCMA (solid line) and isotype-matched control staining (dashed line) revealed BCMA expression on the surface of multiple myeloma cells from a plasmacytoma of myeloma patient 1. The plot is gated on plasma cells, which made up 93% of the total plasmacytoma cells. D, unmanipulated myeloma cells (MM) from a plasmacytoma of myeloma patient 1 or PBMC from myeloma patient 1 were cocultured overnight with allogeneic T cells from myeloma patient 4. The T cells were transduced with either anti-bcma2 or the negative control CAR SP6. The PBMC from myeloma patient 1 did not contain BCMA<sup>+</sup> cells. After the coculture, an IFN-γ ELISA was conducted. E, unmanipulated myeloma cells (MM) from a plasmacytoma of myeloma patient 1 or PBMC from myeloma patient 1 were cocultured overnight with autologous anti-bcma2–transduced T cells or autologous SP6-transduced T cells, and an IFN-γ ELISA was conducted. F, myeloma cells from a plasmacytoma of myeloma patient 1 were specifically killed by autologous anti-bcma2–transduced T cells at low effector:target ratios, whereas autologous SP6-transduced T cells caused only a low level of cytotoxicity of the myeloma cells in a 4-hour cytotoxicity assay. For all effector:target ratios, the cytotoxicity was determined in duplicate, and the results are displayed as the mean ± SEM.
expression in the major human organs. We did not detect BCMA protein expression by any cells except plasma cells (Fig. 2). Normal B-lineage cells are often eradicated from patients receiving infusions of anti-CD19–CAR–expressing T cells (12, 17). Patients lacking B cells can remain free of infections when the patients receive infusions of supplemental immunoglobulins (12, 17), so BCMA expression in normal plasma cells and some normal B cells does not preclude targeting BCMA as a therapy for multiple myeloma.

After showing that BCMA has a limited tissue expression pattern, we designed CARs that incorporated the variable regions of anti-BCMA monoclonal antibodies (Fig. 3). T cells transduced with lentiviral vectors encoding these CARs gained the ability to conduct a variety of in vitro functions in a BCMA-specific manner and to eradicate established BCMA+ tumors in vivo (Figs. 3–5 and Table 1). Both CD4+ and CD8+ T cells exhibited BCMA-specific activation (Fig. 3C and 4A). Compared with CD8+ T cells, a higher percentage of CD4+ T cells produced IL-2 (Fig. 4A). The anti-BCMA-CAR–transduced T cells produced IFN-γ when stimulated with primary multiple myeloma cells and killed primary multiple myeloma cells (Fig. 5).

Evidence that BCMA might be a target for anti-myeloma immunity in humans has been reported (28). Allogeneic stem cell transplantation (AlloSCT) is sometimes used to treat multiple myeloma (4). In patients with relapsed or persistent multiple myeloma after AlloSCT, infusions of unmanipulated allogeneic lymphocytes from the original transplant donor can sometimes induce complete remissions (4, 28). Two patients who obtained complete remissions of relapsed multiple myeloma after infusions of allogeneic donor lymphocytes developed serum anti-BCMA antibodies only after the donor lymphocyte infusions (28). The serum of both of these patients killed BCMAC-expressing target cells in vitro by complement-mediated lysis and antibody-dependent cellular cytotoxicity (28).

In conclusion, BCMA is a promising target for CAR-transduced T cells. We have presented evidence that BCMA is not expressed by normal essential cells, and we detected uniform BCMA protein expression by IHC or flow cytometry on multiple myeloma cells of 5 of 5 patients that were assessed. We designed lentiviral vectors encoding CARs that specifically recognized BCMA. T cells transduced with these CARs gained the ability to carry out BCMA-specific functions. Adoptive transfer of anti-BCMA-CAR–transduced T cells could potentially be an appropriate approach to test in clinical trials enrolling patients with advanced multiple myeloma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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