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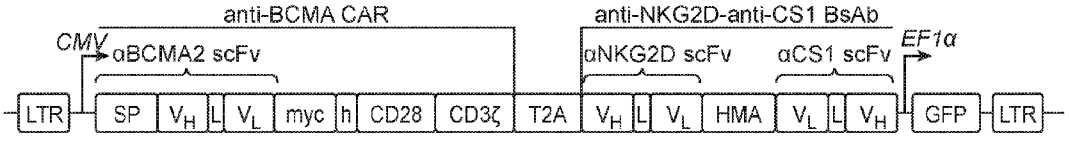


FIG. 3A

(57) Abstract: Described herein are single vectors that, when expressed in cytolytic immune cells, results in *both* the expression of (1) a CAR targeting tumor-associated antigens and (2) secretion of a bispecific antibody that on one end recognizes NKG2D expressed on both innate and antigen specific cytolytic immune cells and on the other end targets tumor associated antigens. Unexpectedly, these modifications to the T cells result in enhanced survival and proliferation *in vivo*. Thus, therapeutic and diagnostic uses are disclosed.

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# BISPECIFIC ANTIBODY CAR CELL IMMUNOTHERAPY

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims priority under 35 U.S.C. § 119(d) to U.S. Provisional Application No. 62/644,343, filed March 16, 2018, the contents of which are hereby incorporated by reference into the present disclosure.

## TECHNICAL FIELD

**[0002]** The present disclosure relates generally to the field of human immunology, specifically cancer immunotherapy.

## BACKGROUND

**[0003]** The following discussion of the background of the invention is merely provided to aid art to the present invention.

**[0004]** Current cancer treatment regimens including chemotherapies, immunomodulatory drugs[14], monoclonal antibodies[15], and autologous or allogeneic transplantation. These therapies often lead to remission, but nearly all patients eventually relapse and succumb to death due to return of the disease. Thus, there is an unmet need for new therapies, including new combination immunotherapies for relapsed and/or refractory disease. This disclosure addresses these limitations and provides related advantages as well.

## SUMMARY OF THE DISCLOSURE

**[0005]** Chimeric antigen receptor (CAR) T cells have been used successfully in the clinic for the treatment of both hematological malignancies and solid tumors, and have recently been approved by the U.S. FDA[1-4]. Bispecific antibodies (BsAb) have also been approved by the FDA for cancer treatment and are being used as an alternative immunotherapeutic approach to CAR T cell therapy[5]. However, CAR- and BsAb-based cancer immunotherapies still need improvement for five important reasons. First, in some cases, CAR T cells cannot be expanded *in vivo* and cannot survive for a sufficient period of time to initiate tumor lysis in patients[6]. It has been reported that the efficacy of CAR T cells correlates with the quantity and duration of CAR T cell presence *in vivo* [6-8]. Second, tumor cells can shed targeted antigens to evade therapy, especially when only a single antigen is targeted. Third, in addition to being costly and time-consuming to manufacture, BsAb have a short half-life and to date have not been shown to be curative[9,10]. Fourth, combination

therapy of CAR T cells with BsAb targeting two distinct tumor associated antigens could be a good approach; however, producing each individually *ex vivo* would be labor intensive and costly; engineering T cells to express *both* a CAR and a BsAb within a *single* construct, of which the BsAB engages *all* cytolytic effector cells has not yet been reported or shown to be additive or synergistic, or to enhance T cell survival *in vivo*. Finally, current approaches to CAR immunotherapy mainly focus on one immune cell type, i.e., T cells, excluding all other cytolytic effector cells in the innate and adaptive arms of the immune system. NKG2D is a c-lectin type of receptor that is expressed on virtually all cytolytic effector cells in both the innate and adaptive arms of the immune system[11,12].

**[0006]** This disclosure provides a platform to resolve these issues, in part or in full, by engineering T cells infected with a single vector delivering these two modes of therapy, i.e., producing a T cell whose CAR is targeting one specific tumor-associated antigen.

**[0007]** Thus, in one aspect, provided herein are chimeric antigen receptors (CARs) comprising, or alternatively consisting essentially of, or yet further consisting of: (a) an antigen binding domain of a cancer or tumor targeting antibody; (b) a hinge domain; (c) a transmembrane domain; and (d) an intracellular domain. Further aspects of the disclosure relate to a chimeric antigen receptor (CAR) comprising, or alternatively consisting essentially of, or yet further consisting of: (a) an antigen binding domain of a tumor targeting antibody; (b) a CD8 α hinge domain; (c) a CD8 α transmembrane domain; (d) a CD28 costimulatory signaling region and/or a 4-1BB costimulatory signaling region; and (e) a CD3 zeta signaling domain.

**[0008]** In certain embodiments, the antigen binding domain of the tumor targeting antibody comprises, or alternatively consists essentially thereof, or further consists of a heavy chain variable region and a light chain variable region that are optionally linked by a linker peptide. In some embodiments, the heavy and/or light chain variable region comprises, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to any one of B-cell maturation antigen (BCMA) and/or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof. In one aspect, the tumor targeting antibody targets BCMA. In some embodiments, the heavy chain and/or light chain variable region comprises, or alternatively consists essentially thereof, or further consists of the amino acid sequence of an antibody to any one of B-cell maturation

antigen (BCMA) and/or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof.

**[0009]** In certain embodiments, the CAR further comprises, or alternatively further consists essentially of, or yet further consists of, a linker polypeptide located between the heavy chain variable region and the light chain variable region. In certain embodiments, the linker is a glycine-serine linker. In further embodiments, the linker polypeptide comprises, or alternatively consists essentially thereof, or further consists of the sequence (glycine-serine)n wherein n is an integer from 1 to 6.

**[0010]** In certain embodiments, the CAR further comprises, or alternatively further consists essentially of, or yet further consists of, a detectable marker and/or a purification marker attached to the CAR.

**[0011]** Additional aspects of the disclosure relate to an isolated nucleic acid sequence encoding a CAR, as described above, or its complement, or an equivalent of each thereof.

**[0012]** In certain embodiments, the isolated nucleic acid sequence further comprises, or further consists essentially of, or yet further consists of, a Kozak consensus sequence located upstream of the polynucleotide encoding the antigen binding domain of a cancer or tumor targeting antibody.

**[0013]** In certain aspects, the isolated nucleic acid further comprises, or alternatively consists essentially thereof, or further consists of a polynucleotide encoding an antibiotic resistance polypeptide operatively coupled to the isolated nucleic acid, a promoter and/or an enhancer element.

**[0014]** Also provided is an isolated nucleic acid encoding a bispecific antibody which recognizes and binds NKG2D. In some embodiments, the nucleic acid encodes a bispecific antibody that comprises, or alternatively consists essentially thereof, or further consists of an NKG2D ligand and, optionally, a SALMF7 (also known as CS1 of CD319) ligand that are optionally codon optimized. In certain embodiments, the isolated nucleic acid encodes a bispecific antibody that comprises, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), that are optionally codon-optimized, or an equivalent of each thereof. In some embodiments, the nucleic acid encodes a bispecific antibody that comprises, or alternatively consists essentially thereof, or further consists of the heavy chain

and/or light chain variable region of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), that are optionally codon optimized, and/or an equivalent of each thereof. In some embodiments, the nucleic acid encodes a bispecific antibody that comprises a single chain variable fragment (scFV) derived from an antibody to NKG2D and, optionally, a single chain variable fragment (scFV) derived from SALMF7 (also known as CS1 of CD319), that are optionally codon optimized and/or an equivalent each thereof. In certain embodiments, the isolated nucleic acid further comprises, or alternatively consists essentially thereof, or yet further consists of a polynucleotide encoding an antibiotic resistance gene.

**[0015]** In further aspect, provided herein are isolated nucleic acids that encode, in one construct, the CAR and bispecific antibody as disclosed above ("BsAb-CAR construct"). In one aspect, the isolated nucleic acid encodes an antigen binding fragment that targets BCMA and a bispecific antibody, e.g., one scFv from an anti-CS1 antibody and one scFv from an anti-NKG2D antibody, joined together by a nucleic acid encoding a non-immunogenic protein linker such as from human muscle aldose. An exemplary BsAb-CAR vector is shown in Figure 3A. The vectors optionally comprise regulatory sequences such as promoters, enhancers, and and viral LTRs.

**[0016]** Aspects of the disclosure relate to a vector comprising one or more of the isolated nucleic acids described above. In certain embodiments, the vector is a plasmid or a viral vector selected from the group of a retroviral vector, a lentiviral vector, an adenoviral vector, and an adeno-associated viral vector. The isolated nucleic acids and vectors containing them are useful to prepare the CARs as described herein.

**[0017]** Further aspects of the disclosure relate to an isolated cell comprising, or alternatively consisting essentially thereof, or further consisting of one or more of the above described compositions: a CAR, an isolated nucleic acid encoding a CAR or its complement, an isolated nucleic acid encoding the CAR/BsA construct, or a vector containing the isolated nucleic acids. In some embodiments, the CAR is expressed on the surface of the isolated cell.

**[0018]** In certain embodiments, the isolated cell further comprises, or alternatively consists essentially of, or yet further consists of, an isolated nucleic acid comprising, or alternatively consisting essentially of, or yet further consisting of a polynucleotide encoding a bispecific antibody, which optionally recognizes and binds NKG2D. In some

embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of an NKG2D ligand or an anti-NKG2 antigen binding fragment of an anti-NKG2 antibody, and, optionally, a SALMF7 (also known as CS1 of CD319) ligand. In certain embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the relevant light chain and heavy chain regions or the CDR regions of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), that are optionally codon-optimized, or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the heavy chain and/or light chain variable region of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), that are optionally codon optimized, and/or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises a single chain variable fragment (scFv) derived from an antibody to NKG2D and, optionally, a single chain variable fragment (scFv) derived from SALMF7 (also known as CS1 of CD319), that are optionally codon optimized and/or an equivalent each thereof. In certain embodiments, the isolated nucleic acid further comprises, or alternatively consists essentially thereof, or yet further consists of a polynucleotide encoding an antibiotic resistance gene.

**[0019]** Non-limiting examples of an isolated cell is a prokaryotic cell such as a bacteria cell, e.g., an *E coli*, or a eukaryotic cell. In some embodiments the isolated eukaryotic cell is selected from an animal cell, a mammalian cell, a bovine cell, a feline cell, a canine cell, a murine cell, an equine cell or a human cell. In further embodiments, the isolated cell is the cell is a T-cell, a B cell, a NK cell, a dendritic cell, a myeloid cell, a monocyte, a macrophage, any subsets thereof, or any other immune cell from any of the species as disclosed herein.

**[0020]** Aspects of the disclosure relate to a composition comprising, or alternatively consisting essentially of, or further consisting of one or more of the above described compositions, e.g., a CAR, an isolated nucleic acid, a cell, or a vector and a carrier.

**[0021]** In certain embodiments, the composition further comprises, or alternatively consists essentially of, or yet further consists of, a bispecific antibody, which optionally recognizes and binds NKG2D, and/or an isolated nucleic acid comprising a polynucleotide encoding a bispecific antibody, which optionally recognizes and binds NKG2D. In some embodiments, the bispecific antibody comprises, or alternatively consists essentially

thereof, or further consists of an NKG2D ligand and, optionally, a SALMF7 (also known as CS1 of CD319) ligand. In certain embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the heavy chain and/or light chain variable region of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises a single chain variable fragment (scFv) derived from an antibody to NKG2D and, optionally, a single chain variable fragment (scFv) derived from SALMF7 (also known as CS1 of CD319), and/or an equivalent each thereof.

**[0022]** Aspects of the disclosure relate to an isolated complex comprising a CAR or a cell comprising the CAR bound to a cancer or tumor antigen or a fragment thereof, and/or a cell expressing the cancer or tumor antigen. In one aspect, the antigen binding domain is expressed on the surface of the cell. In another aspect, the cancer or tumor antigen is B-cell maturation antigen (BCMA), SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof. In one aspect the cell containing or expressing the CAR is a T-cell, a B cell, a NK cell, a dendritic cell, a myeloid cell, a monocyte, a macrophage, any subsets thereof, or any other immune cell. The tumors or cells can be from any animal, e.g., mammalian such as a human cell.

**[0023]** Some aspects of the disclosure relate to a method of producing a CAR expressing cell or a CAR expressing cell that secretes BsA, the method comprising, or alternatively consisting essentially thereof, or yet further consisting of transducing an isolated cell with the nucleic acid sequence encoding a CAR and Bsa or the isolated nucleic acid encoding the BsAb-CAR, as described herein.

**[0024]** In a further aspect, the method further comprises selecting and isolating the cell expressing the CAR or BsAb-CAR. In a further aspect, the cell is a eukaryotic cell such as a mammalian cell, e.g., a human cell such as a T-cell, a B cell, a NK cell, a dendritic cell, a myeloid cell, a monocyte, a macrophage, any subsets thereof, or any other immune cell. The cells can be transduced using the viral vectors as described herein or alternatively using technology described in Riet et al. (2013) Meth. Mol. Biol. 969:187-201 entitled “Nonviral

RNA transfection to transiently modify T cell with chimeric antigen receptors for adoptive therapy."

**[0025]** In certain embodiments, the method further comprises, or alternatively consists essentially of, or yet further consists of transducing the cell with an isolated nucleic acid comprising, or alternatively consisting essentially of, or yet further consisting of a polynucleotide encoding a bispecific anitbody, which optionally recognizes and binds NKG2D. In some embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of an NKG2D ligand and, optionally, a SALMF7 (also known as CS1 of CD319), each optionally codon optimized ligand. In certain embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), optionally codon optimized, or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the heavy chain and/or light chain variable region of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), that are optionally codon optimized and/or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises a single chain variable fragment (scFv) derived from an antibody to NKG2D and, optionally, a single chain variable fragment (scFv) derived from SALMF7 (also known as CS1 of CD319), that are optionally codon optimized, and/or an equivalent each thereof. The cells can be transduced using the viral vectors, e.g., lentiviral vectors, as described herein or alternatively using technology described in Riet et al. (2013) Meth. Mol. Biol. 969:187-201 entitled "Nonviral RNA transfection to transiently modify T cell with chimeric antigen receptors for adoptive therapy."

**[0026]** In certain embodiments, the method of producing a CAR or BsAb-CAR expressing cell further comprises, or alternatively consists essentially of, or yet further consists of activating and expanding the population of CAR expressing cells. Certain aspects of the present disclosure relate to an isolated, activated population of cells comprising, or alternatively consisting essentially of, or yet further consisting of a CAR or BsAb-CAR. In certain embodiments, the cells are one or more of T-cells, B cells, NK cells, dendritic cells, myeloid cells, monocytes, macrophages, any subsets thereof, or any other immune cells.

**[0027]** Aspects of the disclosure relate to a method of inhibiting the growth of a tumor expressing a cancer or tumor antigen, by contacting the tumor with an effective amount of the isolated cells or compositions disclosed above. The contacting can be *in vitro* or *in vivo*. When the contacting is *in vitro*, the method can be used to test personalized therapy against a patient's tumor or to assay for combination therapies. When the contacting is *in vivo*, the method is useful to inhibit the growth of the tumor or cancer cell in a subject in need thereof, such as a human patient suffering from cancer and the patient receives an effective amount of the cells. In certain embodiments, the tumor is a solid tumor. An effective amount is administered alone or in combination with other therapies as described herein. In certain embodiments, the cancer/tumor targeted is a solid tumor or a cancer affecting the blood and/or bone marrow, e.g., multiple myeloma (MM). In certain embodiments the isolated cells are autologous to the subject being treated. In aonther aspect, the cells are allogeneic to the subject being treated. In another aspect, the method further comprises, or consists essentially of, or yet further consists of, administering to the subject an effective amount of a cytoreductive therapy. In a further aspect, the method further comprises the steps of isolating the cells to be administered to the subject, transducing the cells with an effective amount of an isolated nucleic acid encoding a CAR or BsAb-CAR as described herein, culturing the cells to obtain a population of CAR or BsAb-CAR encoding cells, that are optionally expanded and activated and then administering the cells to the patient.

**[0028]** Also disclosed herein are kits comprising one or more of the above noted compositions and instructions for their use in the methods as disclosed herein.

**[0029]** The above compositions and methods are unique and overcome the limitation of the state of the art in that they provide a CAR cell that simultaneously secrets a NKG2D-based BsAb targeting a second tumor-associated antigen. The disclosed CAR NKGD2D-based BsAb is exemplary only. This approach can be modified for any number of tumor antigens, as know in the art, e.g., EGFRVIII; CD70, mesothelin, CD123, CD19, CEA, CD133, Her2, see Townsend et al. (2018) J. Exp. & Clinical Cancer Res. 37:163.

**[0030]** The exemplary constructs were tested in a multiple myeloma model (MM). MM is a malignancy characterized by an accumulation of clonal plasma cells[13]. As noted above, current treatment regimens including chemotherapies, immunomodulatory drugs[14], monoclonal antibodies[15], and autologous or allogeneic transplantation often lead to remission, but nearly all patients eventually relapse and succumb to death due to return of

the disease. Thus, there is an unmet need for new therapies, including new combination immunotherapies for relapsed and/or refractory MM.

**[0031]** As a component of the innate immune system, natural killer (NK) cells play an important role in preventing tumor growth[16] , but NK cell anti-tumor activity has been found to be dampened in many MM patients[17]. Adoptive transfer of activated or allogeneic NK cells produce effective anti-tumor responses in the treatment of a number of hematological malignancies, including MM[18,19], and solid tumors. However, in many cases, NK cell–mediated antitumor responses are weak, which may result from NK cell expression of inhibitory receptors, poor capacity for survival, or limited migration of effector cells into tumor sites [20-22]. Meanwhile, as a part of adaptive immunity, T cells can migrate efficiently into various tissues, and tend to proliferate well in response to antigen stimulation. However, T cells have strict specificities dictated by antigen-specific T-cell receptors (TCR). Thus, a method to engage both T cells and NK cells, and thus overcome their own limitations would be of great benefit for cancer immunotherapy. This disclosre provides this benefit.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0032]** **FIGs. 1A-1D** show the results of engineering T cells to express BCMA CAR and anti-NKG2D-anti-CS1 bi-specific fusion protein individually or in combination. (A) Schematic representation of the BCMA CAR lentiviral constructs containing a scFv against BCMA linked to CD28 and CD3zeta(ζ) endodomains. The expression of the transgene was traced by GFP expression driven by an EF1alpha(α) promoter. LTR, long terminal repeats; SP, signal peptide; VH, variable H chain; L, linker; VL, variable L chain. MyC, MyC tag; Hinge, Hinge Chain; CD28, a T cell co-stimulatory molecule; CD3ζ, CD3 zeta chain. (B) Schematic diagram of lentiviral construct for mammalian expression of anti-NKG2D-anti-CS1 bispecific antibody (BsAb). The anti-NKG2D-anti-CS1 BsAb consisted of an anti-NKG2D scFv, which was composed of VH and VL linked together by a linker (L), and an anti-CS1 scFv. Expression of the BsAb is driven by a CMV promoter flanked by lentiviral LTR. (C) PBMC (peripheral blood mononuclear cells) from healthy donors were activated with CD3 and CD28 beads and transduced with the pCDH empty vector (EV), BCMA CAR, anti-NKG2D-anti-CS1 BsAb. The activated T cells were also sequentially transduced with BsAb and BCMA-CAR and these transduced cells were named “BsAb-BCMA seq. trans. T”. GFP-positive cells were sorted, and cells were stained with biotin labeled goat

anti-mouse Fab specific or isotype-matched control antibody, followed by streptavidin and CD3 antibody staining. (D) Supernatant of unmodified T cells, BsAb T cells or BsAb-BCMA seq. trans. T cells were collected, and individual cell lysates were subjected to immunoblot analysis with anti-6x His–tag antibody.

**[0033]** **FIGs. 2A-2E** show that BsAb-BCMA seq. trans. T cells possess higher capacity of cytotoxicity and IFN-gamma(γ) production than BCMA-CAR T cells or BsAb T cells in response. (A) Flow cytometric analysis of BCMA and CS1 expression on the surface of MM cell lines. Three MM cell lines (MM1.S, H929, and RPMI-8226) and one chronic myelogenous leukemia cell line (K562) were stained with anti-CS1 mAb antibody (upper panel) or anti-BCMA mAb (lower panel), different colors were used to distinguish the three MM cell lines, MM.1S (green), H929 (red), and RPMI-8226 (gray) as well as the K562 cell line (blue) or isotype-matched control antibody (black solid line and open area). (B) $^{51}$Cr-labeled MM1.S, H929, RPMI-8226 MM cell lines and the K562 cell line ($5 \times 10^3$ for each cell line) were co-cultured with unmodified T cells (T, black solid line), empty vector-transduced T cells (EV T, black dotted line), T cells expressing anti-NKG2D-anti-CS1 BsAb (BsAb T, red solid line), BCMA CAR (BCMA CAR T, green solid line), and BsAb-BCMA seq. trans. T cells (blue solid line) at the indicated E:T ratios for 4 hours, and target lysis ($^{51}$Cr release) was measured. BsAb-BCMA seq. trans. T vs BCMA CAR T, * $p<0.05$, ** $p<0.01$; seq. trans. T vs BsAb T, # $p<0.05$, ## $p<0.01$. K562 cells as $BCMA^{-}CS1^{-}$ negative control. (C) unmodified T cells (white square), EV T cells (gray shadow square), BsAb T cells (red square), BCMA CAR T cells (green square) or BsAb-BCMA seq. trans. T cells (blue square) $2 \times 10^5$ were cultured alone (no target) or stimulated with an equal number of MM.1S, H929, or RPMI-8226 MM cells expressing different levels of CS1 and BCMA or $BCMA^{-}CS1^{-}$ K562 cells for 24 hours, and the supernatants were collected to measure IFN-γ secretion by ELISA. * $p<0.05$, ** $p<0.01$, n.s. no significant difference. (D and E) Cells were treated as described in (c), and IL-2 or TNF-alpha(α) secretion in cell-free supernatants was determined by ELISA, respectively. ** $p<0.01$, n.s. no significant difference.

**[0034]** **FIG. 3A-3H** show the generation of a BsAb-CAR vector containing both BCMA CAR and ant-NKG2D-anti-CS1 bispecific antibody (BsAb) in the same construct and functional examination of T cells transduced with this construct. (A) Schematic representation of a generated lentiviral vector expressing both BCMA CAR and anti-

NKG2D-anti-CS1 BsAb (referred heretoafter as BsAb-CAR). T2A, a self-cleaving 2A gene. (B) Supernatants and cell lysates of empty vector (EV)-tranduced T cells or BsAb-CAR T cells were subjected to immunoblot analysis with an anti-6x His-tag antibody. (C) $^{51}$Cr-labeled MM1.S cells ($5 \times 10^3$) were co-cultured with unmodified T cells (T, black solid line), empty vector-transduced T cells (EV T, black dotted line) or BsAb-CAR T cells (purple line) at the indicated E:T rations for 4 hours, and target lysis ($^{51}$Cr release) was measured. (D) Unmodified- (black solid line), EV- (black dotted line), BsAb- (red line), BCMA CAR- (green solid line) or BsAb-CAR (purple solid line)-transduced CD8 (+) T cells isolated from healthy donors were co-cultured with $^{51}$Cr-labeled MM1.S MM cells ($5 \times 10^3$) at the indicated E:T ratios for 4 hours, and target lysis ($^{51}$Cr release) was measured. (E) 4-hour $^{51}$Cr release assays with MM.1S MM cells at an E:T ratio of 10:1. To assess this anti-tumor effect in the presence of different quantities of normal (uninfected) human PBMCs, PBMCs were added at a quantity of 1-fold, 10-fold, 100-fold, and 200-fold of the MM.1S MM target cells. (F) $^{51}$Cr release assays of unmodified T cells (black square), EV T cells (pattern square), BsAb T cells (red square), BCMA-CAR T cells (green square), or BsAb-CAR T cells (purple square) against MM.1S MM target cells at an E:T ratio of 5:1. As noted in the figure, the incubation time of effector cells and MM.1S MM target cells is 4-hr for PBMC, NK and NKT cells (left panel) and 16-hr for $CD3^+$T cells, $CD8^+$T cells, $V\gamma9V\delta2$ T cells or $CD4^+$T cells. * $p<0.05$, ** $p<0.01$, n.s. no significant difference. (G) Control of co-culture of EV T cells (GFP, green) and MM.1S MM cells (red) after 1 hour, confocal microscopy analysis of synapses was determined (scale 10μL, upper panel; scale 20μL, lower panel); No synapses are noted, even at higher power shown in the lower panel. (H) One hour co-culture of BsAb-CAR T cells (E: GFP, green) and MM.1S MM cells (T: red); E/T synapses were observed and indicated by the arrows in all frames (S1 shows the same conjugated E/T pair in each frame moving left to right, as does S2, and S3). The frame on the top, left shows a bright field (Bf, scale 10μL); the top, middle frame shows an immunofluorescence image of BsAb-CAR T cells (GFP, green) and MM.1S MM cells (red) co-culture (scale 10μL). The frame on the top, right is a merged image with additional anti-6x-His-tag identifying the BsAb (blue, scale 10μL). The bottom three rows demonstrate the three individual E/T conjugates (S1, S2, and S3) visualized in an enlarged field (scale 20μL).

**[0035]** **FIGS. 4A-4D** show that overexpression of BCMA and CS1 in K562 cells triggers enhanced cytotoxicity and cytokine secretion after recognition by BsAb-CAR T cells. (A)

Flow cytometric analysis of K562 cells overexpressing CS1 and BCMA (K562-CS1-BCMA, gray shadow) or an empty vector control (K562-PCDH, black solid line) after the cells were stained with a CS1 (left panel) or BCMA (right panel) or IgG isotype control (black dotted line in each panel) antibody. (B) Cytotoxicity of empty vector (EV)- or BsAb-CAR-transduced T cells against K562-CS1-BCMA and K562-PCDH cells, determined by 4-hour $^{51}Cr$ release assays. K562-CS1-BCMA or K562-PCDH cells were incubated with EV T cells or BsAb-CAR T cells at indicated E:T ratios. ** $p<0.01$ (K562-CS1-BCMA + BsAb T cells vs. K562-PCDH + BsAb T cells). (C) EV T cells or BsAb-CAR T cells ($1 \times 10^5$) were cultured alone or stimulated with an equal number of either K562-CS1-BCMA or K562-PCDH cells. Supernatants from cultures were used to determine IFN-γ secretion by ELISA. ** $p<0.01$. (D) Cells were treated as in (C) and IL-2 secretion in cell-free supernatants was determined by ELISA. ** $p<0.01$.

**[0036] FIGS. 5A-5E** show that secreted anti-NKG2D-anti-CS1 BsAb enhances CAR T cell proliferation through NKG2D signaling. (A) Medium color following culture of unmodified T cells (1), 2-EV T cells (2), BsAb T cells (3), BCMA CAR T cells (4), BsAb-CAR T cells (5) or naïve T cells (6) (non-proliferate control) were displayed in upper panel. The bar graph provides statistical analyses of total cell number included 6 individual samples for each group. ** $p<0.01$ (group 5 vs. groups 1, 2, 4 and group 3 vs. groups 1, 2, 4). To document T cell proliferation or lack thereof, violet cell tracker was used and shown as V450 dilution that is displayed by histograms in the lower panel. (B) Five day-old culture medium of unmodified T cells, EV T cells, and BCMA CAR T cells in the presence or absence of cell-free supernatants of BsAb-CAR T cells from (A), designated as 1+, 2+, 4+ or 1, 2, 4, respectively. Cells were enumerated, and data were presented as a bar graph (top). ** $p<0.01$ (4+ vs. 4, 2+ vs. 2, 1+ vs. 1). Violet cell tracker was shown as V450 dilution that displayed by histograms in the lower panel (bottom). (C) Two-day-old culture medium is shown. 1A-unmodified T cells, 2A-EV T cells, 3A-BsAb T cells, 4A-BCMA CAR T cells, and 5A-BsAb-CAR T cells. On day 0, NKG2D blockade antibody (20 μg/mL) was added into culture of 1B, 2B, 3B, 4B and 5B, while a nonreactive isotype control antibody (20 μg/mL) was added to 1A, 2A, 3A, 4A and 5A). Beneath these wells is flow cytometric staining for CD3, NKG2D, $F(ab)_2$ (which is denoted by "Fab" indicating expression of the CAR), and Ki67 to measure the cell proliferation. (D) Immunoblot analysis was performed to determine the phosphorylation (p) of AKT protein, and total AKT protein of 1A---Unmodified T, 2A---EV T, 3A---BsAb T, 4A---BCMA-CAR T, and

5A---BsAb-CAR T as well as 1B-Unmodified T cells + NKG2D blockade, 2A---EV T+ NKG2D blockade, 3A---BsAb-T+NKG2D blockade, 4A---BCMA-CAR T+NKG2D blockade, and 5A---BsAb-CAR T+NKG2D blockade. (E) The same cells shown in (C) (1A, 2A, 3A, 4A and 5A) were also co-cultured with MM.1S MM cells for 48 hours. Flow cytometric analyses to assess cell proliferation was performed as described above in (C).

**[0037]** **FIGS. 6A-6C** show that secreted anti-NKG2D-anti-CS1 BsAb enhances CAR T cell survival through NKG2D signaling *in vitro*. (A) Five day-old culture media of 1---Un. (Unmodified) T + IL-2, 2---EV T + IL-2, 3---BsAb T + IL-2, 4---BCMA-CAR T + IL-2, 5---BsAb-CAR T + IL-2 were displayed. Flow cytometric staining for CD3 ($1^{st}$ column), $F(ab)_2$ ($2^{nd}$ column), and Ki67 to observe the cell proliferation ($3^{rd}$ column), and Annexin V/Sytox Blue to observe the cell survival ($4^{th}$ column). (B) Five day-old culture media (without IL-2) of 1---unmodified T, 2---EV T, 3---BsAb T, 4---BCMA-CAR T, and 5---BsAb-CAR T were shown in the upper panelze. Flow cytometric staining for CD3 ($1^{st}$ column) and Ki67 ($2^{nd}$ column) to detect the cell proliferation. Annexin V/ Sytox Blue was included to detect cell survival ($3^{rd}$ column). (C) Statistical analyses of percentages of CD3, Ki67 proliferative cells, Annexin V(-)Sytox Blue(-) alive cells, Annexin V(+) apoptosis cells, and Annexin V(+)Sytox Blue(+) dead cells were displayed. Multiple *t*-test, compared each groups. ** $p<0.01$.

**[0038]** **FIGS. 7A-7D** show BsAb-CAR transduced-T cells have better proliferation and survival capacity than BCMA-CAR T cells and control T cells *in vivo*. (A) Design of i.v. injection of unmodified T cells, EV T cells, BCMA-CAR T cells and BsAb-CAR T cells into immunodeficient NSG mice (a, upper). 3D histograms (lower panel, $1^{st}$ column) indicate the percentages of injected human CD3 T cells. The blue histograms are for the mice that had no T cell injection on day -1, the orange histograms represent 1 day after T cell injection, and the black histograms represent 14 days after T cell injection (red arrow points the BsAb-CAR T group). Contours indicate CD69 expression (orange for 1 day after i.v. injection, and black for 14 days after i.v. injection). The purple color histograms ($4^{th}$ column) indicated percentages of the injected CD3 T cells 35 days after i.v. The purple contours are the combination of Ki67 and CD69 staining of 4 groups to reveal the cell proliferation. The red contours are the combination of Sytox Blue and Annexin V staining to reveal the cell apoptosis and cell death. S-/A- denotes Sytox Blue (-)/Annexin V(-), S-/A+ denotes Sytox Blue-/Annexin V+, and S+/A+ denotes Sytox Blue+/Annexin V+. (B, C)

Statistical analysis of human $CD3^+$ (B) and $CD69^+$ (C) cells shown in (A). Unmodified T (empty rectangle), EV T (pattern filled rectangle), BCMA-CAR T (gray shadow rectangle), and BsAb-CAR T (black rectangle). Multiple t-test, compared each group. ** $p<0.01$, n.s. no significant difference, n=5 mice per group. (D) Statistical analysis of the percentages of $Ki67^+CD69^+$ proliferative cells, Annexin V(-) Sytox Blue(-) alive cells, Annexin V(+)Sytox Blue(-) apoptosis cells, and Annexin V(+)Sytox Blue(+) dead cells. Multiple t-test, compared each group. ** $p<0.01$, n.s. no significant difference, n=5/ group.

**[0039]** **FIGS. 8A-8C** show BsAb-CAR T cells specifically recognize and eliminate CS1 or/and BCMA-expressing human primary multiple myeloma cells *ex vivo*. (A) Flow cytometric surface staining for CS1 and BCMA protein in $CD138^+$ multiple myeloma tumor cells isolated from MM patients' bone marrow. Results from 8 patients are shown. Eight patients' MM cells were stained with PE-conjugated anti-CS1 mAb antibody (left panel) or APC-conjugated streptavidin with biotin-labeled anti-BCMA mAb (right panel). Various colors are used to indicate each of the 8 patients or isotype-matched control antibody (gray shadow). (B) $5 \times 10^3$ $CD138^+$ multiple myeloma tumor cells were co-cultured with EV T cells (black dotted line), BsAb T cells (red line), BCMA-CAR T cells (green line), or BsAb-CAR T cells (purple line) at the indicated E:T ratios for 4 hours, then specific lysis was determined using a standard $^{51}Cr$ release assay. Representing data from Patient sample 1 and Patient sample 4 are shown. Eight patients' data were analyzed and shown as individual values for each pateint (right panel). (C) Transduced T cells as indicated were co-cultured with $CD138^+$ multiple myeloma tumor cells at an E:T ratio of 1:1 for 24 hours, and IFN-$\gamma$ secretion was measured in cell-free supernatants via ELISA.

**[0040]** **FIGS. 9A-9C** show that BsAb-CAR T cells are superior to suppress *in vivo* MM growth and prolong survival of mice bearing MM or being re-challenged with tumor cells. (A) Bioluminescence imaging was shown for five representative mice bearing MM.1S tumors from each indicated group. NSG mice were intravenously inoculated with $8 \times 10^6$ MM.1S cells expressing luciferase (day 0). On days 10, 17 and 24 after tumor implantation, each mouse received an i.v. injection with either saline (control group) or, $10 \times 10^6$ EV T cells, BsAb T cells, BSMA CAR T cells, BsAb-BCMA seq. trans. T cells, or BsAb-CAR T cells, respectively (upper panel, experiment schedule). Images on the row were taken on day 10 after tumor implantation, just before infusion of engineered T cells or control T cells. Images in the middle row were taken on day 24, after mice already undergone treatment

twice (on day 10, 17) and just prior to the third treatment. Images in the bottom row show mice on day 31, after 3 rounds of treatment (on day 10, 17, and 24). (B) On day 80 after tumor implantation, peripheral blood (PBL) were collected from survived mice (3 mice of BCMA CAR T cells treated group, 4 mice of BCMA seq. trans. T cells treated group, and 5 mice of BsAb-CAR T cells treated group). PBL total cell numbers were calculated (left panel). Flow cytometric staining with FITC-conjugated anti-human CD45 mAb antibody and APC-conjugated streptavidin with biotin-labeled goat anti-mouse $(Fab)_2$ polyclonal antibody or normal polyclonal goat immunoglobulin G (IgG) antibody. The percentages of Fab(+) cells and numbers were calculated as shown in the middle. (C) Kaplan-Meier survival curve of MM.1S-bearing mice treated with various transduced-T cells, saline (black solid line), EV T cells (black dotted line), BsAb T cells (red line), BCMA CAR T cells (green line), BCMA seq. trans. T cells (blue line), and BsAb-CAR T cells (purple dotted line). The gray dotted vertical line with arrow indicated the day 80 when mice were re-challenged with $4 \times 10^6$ MM.1S cells.

**[0041] FIGS. 10A-10D** show that BsAb-CAR T cells more effectively than BCMA-CAR T cells suppress *in vivo* MM growth and prolong survival of MM tumor-bearing mice in the presence of adoptively transferred human PBMC. (A) Bioluminescence imaging was shown for three representative mice bearing MM.1S tumors from each indicated group. NSG mice were intravenously inoculated with $8 \times 10^6$ MM.1S cells expressing luciferase (day 0). On days 10, 17 and 24 after tumor implantation, each mouse received an i.v. injection with either saline (control group), BSMA-CAR T cells, or BsAb-CAR T cells. Myeloid cells-depleted PBMC from the same donor were i.v. injected to mice on day 10 after tumor implantation (upper panel, experiment schedule). Images on the first column were taken on day 10 after tumor implantation, just prior to infusion of engineered T cells or control T cells. Images in the second column were taken on day 19, after the mice already undergone treatment twice (on day 10, 17) and just before the third treatment was administered. Images in the third column show mice on day 28, after 3 rounds of treatment (on day 10, 17, and 24). Images in the fourth column show mice on day 37. (B) Blood was collected from survived BsAb-CAR T cells injected mice (n=5), and untreated NSG mice (None, n=5). Flow cytometric staining for CD19/20(+) human plasma cells, CD56(+) NK cells, and CD3(+) T cells. Green color of contour that gated on human CD3(+)$F(ab)_2$(+) indicate the percentage of CAR expression within survived human T cells. (C) Statistical analysis of the percentages of human CD19/20(+) plasma cells, CD56(+) NK cells, CD3(+) T cells, and

CD3(+)F(ab)$_2$(+) survived CAR T cells. (d) Kaplan-Meier survival curve of MM.1S-bearing mice treated with various transduced-T cells, saline (black solid line), BCMA-CAR T cells (green line), or BsAb-CAR T cells (purple dotted line). $P < 0.0001$, BsAb-CAR T cells vs. BCMA-CAR T cells.

**[0042] FIGS. 11A-11C**. (A) 3D rainbow dots flow cytometric map (basic on CD3 staining, CD3 positive cells show the yellow and green color, and CD3 negative cells show the dark blue and purple color) displays the percentages of CD3(+) T cells (black circle, yellow color with green color), γδ T cells (yellow color alone), NKT cells (orange circle, yellow color with green color), and NK cells (blue circle, dark blue and purple color). 2D contour maps show the details of 3D map. (B) Flow cytometric staining of NKG2D surface expression in T cells, CD8$^+$ T cells, pan γδ T cells, Vγ9Vδ2 T cells, NKT cells, and NK cells. Data presented are representative of PBMC from 10 healthy donors. (C) Statistical analyses of percentages of NKG2D$^+$ cells in (B). n=10.

**[0043] FIGS. 12A-12B**. (A) 4-hour 51Cr release assays at the E:T ratio of 10:1 [E, effector cells of unmodified T cells (black solid line) or EV- (black dotted line), BsAb- (red line), BCMA-CAR- (green line), or BsAb-CAR-transduced T cells (purple line)]. Different quantities of human PBMC at 1-fold, 10-fold, 100-fold, or 200-fold over target cells. Sspecific lysis curve of one representative experiment of three are shown in A and the summary data of three are shown in B. (B) Statistical analyses of $^{51}$Cr release assays results of (a), multiple t-test, * $p<0.05$, ** $p<0.01$, n.s. no significant difference, 3-time repetition.

**[0044] FIGS. 13A-13D**. (A) CD3(+) T cells, CD8(+) cytotoxic T cells, CD4(+) T cells, γδ T cells, NKT cells, and NK cells were isolated from leukopacks ordered from the American Red Cross. Black color contour map for primed T shows combinational staining of CD3 and pan αβ TCR. Brown and green color contour maps show sorted CD8(+) and CD4(+) T cells, respectively. (B) Activated human NK cells were stauined with CD3 and CD56. (C) Sorted pan γδ T cells were stained with CD3 and pan γδ TCR antibodies. Black color contour maps for activated Vγ9γδ2 TCR T cells were performed by combinational staining of CD3, CD56, Vγ9 and Vδ2. (D) Freshly FACS-sorted human CD3(+)CD56(+) NKT cells were stained with CD3 and CD56.

**[0045] FIGS. 14A-14E**. (A) $^{51}Cr$ release assays of unmodified T cells (black solid line), EV T cells (black dotted line), BsAb T cells (red line), BCMA-CAR T cells (green line) or BsAb-CAR T cells (purple line) at an E:T ratio of 5:1 at different time points, including 2h, 4h, 8h, and 16h. No additional PBMC were added. (B, C, D, E) Above described cytotoxicity assays were repeated in the presence or absece of bulk T cells or individual T cell subsets including primed CD3(+)T cells, CD8(+)T cells, CD4(+)T cells, and Vγ9Vδ2 T cells. The ratio of effector cells to target MM.1S cells is 5:1 for all experiemnts.

**[0046] FIGS. 15A-15J** show confocal microscopic analysis following 24 hour co-culture of either BsAb-CAR T cells (green) or EV T cells (green) with MM.1S MM cells (red). (A-F) Co-culture of BsAb-CAR T cells with MM.1S MM cells for 24 hours shows elimination of MM.1S MM cells. (G-J) Co-Culture of EV T cells with MM.1S MM cells for 24 hours shows persistence of MM.1S MM cells. Bf = Bright field; Scale, 10μL.

**[0047] FIG. 16** shows the generation of K562 cells stably expressing the CS1 and BCMA genes. The left pseudo color flow map indicates the control of un-transduced K562 cells. The middle pseudo color flow map indicates FACS-sorted pCDH-CS1-GFP lentivirus-infected K562 cells. The third pseudo color flow map indicates FACS-sorted CS1(+)BCMA(+) K562 cells.

**[0048] FIGS. 17A-17D**. (A) 48 hours' culture. 1A-unmodified T cells, 2A-EV T cells, 3A-BsAb T cells, 4A-BCMA CAR T cells and 5A-BsAb-CAR T cells. Flow cytometric staining for CD3 and NKG2D to observe CD3(+) populations (blue flames), CD3(+)NKG2D(+) populations (red flame), and CD3(+)NKG2D(-) populations (green flames). (b) On day 0, NKG2D blockade antibody (20 μg/mL) was added in to (A) culture and named as 1B, 2B, 3B, 4B and 5B. After 48 hours, flow cytometric staining for CD3 and $F(ab)_2$ to observe the cell population. CD3(+) populations (blue flames). (C) 48 hours' culture media from (A) were shown. On day 0, CS1 blocking antibody (20 μg/mL) was added into (A) culture and named as 1C, 2C, 3C, 4C and 5C. After 48 hours, flow cytometric analysis was performed after for staining cells with anti-CD3, anti-$F(ab)_2$, anti-NKG2D, and anti-Ki67 to observe cell proliferations. Cells were gated on CD3(+) and CD3(+)NKG2D(+) populations (red flame), CD3(+)NKG2D(-) populations (green flames) were indicated. Red color dots flow maps indicated the proliferation of NKG2D(+)Fab(+) (upper three) or NKG2D(+)Fab(-) (lower two) cells. Green color dots flow maps indicate proliferation of NKG2D(-)Fab(+) (upper three) or NKG2D(-)Fab(-) (lower two) cells. Fab

denotes anti-F(ab)$_2$ stianing for CAR expression. (D) Cells from 1A, 2A, 3A, 4A and 5A in (A) were co-cultured with MM.1S tumor cells for 48 hours. Flow cytometric analyses were performed after staining cells with anti-CD3, anti-F(ab)2, and anti-NKG2D antibodies. Fab denotes F(ab)2 staining, which indicates CAR expression.

**[0049] FIG. 18** provides supplemental data to the data in FIG. 7. In FIG. 7, 3D histograms (1$^{st}$ column) of human CD3 T cells percentages are shown. These are the original pseudo color flow maps. The blue color frame indicated the human CD3 percentages of the mice that had no T cell injection on day -1 (1$^{st}$ column). The orange color frame represents anti-human CD3(+)cells in mice one day after T cell injection, and CAR expression were detected by flow cytometric anlysis after staining with anti-F(ab)$_2$ (2$^{nd}$, 3$^{rd}$ column). The black color frame shows anti-human CD3(+) cells in mice 14 days after infusion with enginerred or control human T cell (4$^{th}$ column). The purple color frame shows anti-human CD3(+) cells in mice 35 days after infusion with enginerred or control human T cell (5$^{th}$ column). Statistical analysis for CD3(+) human cells is shown in Figure 7.

**[0050] FIGS. 19A-19B** show the effects of depletion of myeloid cells in human healthy donor's PBMC to avoid GVHD when injected to NSG mice. (A) Ficoll-Paque PLUS isolated human PBMC were stained. Number 1 indicated lymphocyte percentages, 2 indicated granulocyte percentages, and 3 indicated monocyte percentages. CD11c, CD14, CD33 and CD66b were stained to check the percentages of myeloid cells in healthy donor's PBMC before FACS sorted. (B) After sorting, CD11c, CD14, CD33 and CD66b were stained as above described, pseudo color low maps were displayed.

**[0051] FIG. 20** provides an assessment of cytotoxicity of human BsAb-CAR T cells against autologous PBMC, T cells, NK cells, and plasma cells by a standard 4h-$^{51}$Cr release assay. PBMC were isolated by Ficoll-Paque PLUS gradient centrifugation. CD3(+) T cells, CD56(+) NK cells, and CD19/20(+) plasma cells were FACS-sorted from PBMC. EV T cells were used as control in the cytotoxicity assay.

**[0052] FIGS. 21A-21D**. (A) Different time points (12h, 24h, 48h, 72h, and 96h) of BsAb BCMA-CAR (BsAb-CAR T) lentivirus-infected healthy donor's primed T cells. The same view of BF (Bright field) GFP (green) is shown. (B) Immunoblotting with anti-His-tag to show secretion of BsAb in the supernatant of BsAb-CAR T cells. (C) Flow cytometric staining for BsAb-CAR T lentivirus- infected primed T cells. GFP-positive cells were sorted, and cells were staines with biotin labeled goat anti-mouse Fab specific or isotype-

matched control antibody, followed by streptavidin and CD3 antibody staining. (D) $^{51}$Cr-labeled H929, RPMI-8226 and K562 target cell lines ($5 \times 10^3$) were co-cultured with unmodified T cells (black solid line), empty vector-transduced T cells (EV T, black dotted line), or BsAb-CAR T cells (purple line) at the indicated E:T ratios for 4 hours. Target lysis ($^{51}$Cr release) was measured. BsAb-CAR T vs unmodified T or EV T, ** $P$<0.01, Repeated for three times. BCMA(-)CS1(-) negative K562 served as negative control target cells.

## DETAILED DESCRIPTION

**[0053]** It is to be understood that the present disclosure is not limited to particular aspects described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims. Throughout this disclosure, various technical publications are referenced by an Arabic numeral. The complete citaitons for these publications can be found immediately preceding the claims and are incorporated herein by reference.

**[0054]** Both B-cell maturation antigen (BCMA) and SLAMF7 (CS1 or CD319) have been shown to be excellent MM tumor antigen targets, while NKG2D is a superb immune cell target. NKG2D, a receptor, is expressed on virtually all cytolytic immune cells, including NK cells, NKT cells, CD8(+) T cells, and γδ T cells [23]. BCMA is a member of the tumor necrosis factor receptor superfamily (TNFRSF17 or CD269), is selectively induced during plasma cell differentiation and is nearly absent on naïve and memory B cells [24,25]. Adoptive transfer of anti-BCMA-CAR-expressing T cells has been reported as a promising new strategy for treating MM[26-28]. CS1 is another attractive tumor-associated target antigen in MM, because CS1 is highly and ubiquitously expressed on the surface of MM cells[29,30]. CS1 is expressed at low levels on NK cells and on a subset of activated CD8(+) T cells, but it is almost undetectable on myeloid cells and normal hematopoietic stem cells[31]. A therapeutic monoclonal antibody against CS1 has been approved by the FDA for the treatment of MM[32]. Applicant's published research showed that genetic modification of T cells or NK cells redirected toward CS1 enhanced eradication of myeloma cells[29,30]. A recent preclinical study showed that patient MM cells subjected to CS1 CAR T cell treatment were effectively eradicated, while normal NK and T cells with low levels of CS1 expression remained unaffected (Gogishvili, 2017 Blood. 2017 Dec 28;130(26):2838-2847. doi: 10.1182/blood-2017-04-778423. Epub 2017 Oct 31). NKG2D, an activating receptor,

is expressed on a variety of innate and adaptive cytolytic cells as mentioned above [11,33]. Triggering NKG2D can lead to activation of both innate and adaptive cellular immunity[34].

**[0055]** As disclosed herein, Applicants engineered T cells to (1) express a BCMA-specific second-generation CAR and (2) simultaneously secret an anti-NKG2D-anti-CS1 BsAb. These data suggest that these cells, hereafter referred to as BsAb-CAR T cells, represent a promising therapy for relapsed and/or refractory MM, and can be a suitable platform for producing the next generation CAR-based cancer immunotherapy.

**[0056]** Such a combination of a CAR and an anti-NKG2D-based bispecific antibody as a single vector transduced into T cells has yet to be described in the literature. Applicants have demonstrated that this approach generates T cells (BsAb CAR T-cells) that function as potent cytolytic effector cells against tumors. Provided herein is a representative example of a T cell CAR is directed against a well-known target in multiple myeloma (MM), called BCMA, and the anti-NKG2D-based bispecific antibody recognizes the well-described MM tumor antigen CS1 that brings it into close proximity of any innate or adaptive cytolytic effector cell bearing the NKG2D antigen. However, contemplated herein is the expansion to variety of complementary CARs and anti-NKG2D-based bispecific antibodies into a single vector to then infect T and NK cells for anti-tumor efficacy. This approach can be modified for any number of tumor antigens, as know in the art, e.g., EGFRVIII; CD70, mesothelin, CD123, CD19, CEA, CD133, Her2, see Townsend et al. (2018) J. Exp. & Clinical Cancer Res. 37:163.

**[0057]** As shown in the examples below, a single single vector delivering two complementary modalities directed against two distinct tumor associated antigens on MM cells, is superior to either modality alone and is superior to the use of T cells infected sequentially with the two separate constructs, one encoding the CAR and the other encoding the anti-NKG2D-based bispecific antibody. Further, when the T cell (infected with the experimental vector encoding both the CAR and the anti-NKG2D-based bispecific antibody) encounters the MM cell expressing both antigens, the BsAb CAR T cell undergoes both proliferation and enhanced survival *in vitro* and *in vivo* through NKG2D-mediated activation. Such results are altogether unexpected.

**[0058]** In sum, applicants constructed BCMA CAR T cells that also secrete anti-NKG2D-anti-CS1 bispecific antibodies; these cells effectively target BCMA(+) and/or CS1(+) multiple myeloma (MM) cells. The secretion of anti-NKG2D-anti-CS1 bispecific

antibodies by BCMA CAR T cells enhances both CAR T cell proliferation in vitro and CAR T cell survival and proliferation *in vivo* through NKG2D-mediated activation. BCMA CAR T cells secreting anti-NKG2D-anti-CS1 bispecific antibodies display significantly better *in vitro* and *in vivo* efficacy against tumor cell targets compared to single therapies with BCMA CAR T or with T cells secreting anti-NKG2D-anti-CS1 bispecific antibody alone. These results can be generalized to a variety of CARs employed in the same manner.

**[0059]** Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this technology belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present technology, the preferred methods, devices and materials are now described. All technical and patent publications cited herein are incorporated herein by reference in their entirety. Nothing herein is to be construed as an admission that the present technology is not entitled to antedate such disclosure by virtue of prior invention.

**[0060]** The practice of the present technology will employ, unless otherwise indicated, conventional techniques of tissue culture, immunology, molecular biology, microbiology, cell biology, and recombinant DNA, which are within the skill of the art. *See,* e.g., Green and Sambrook eds. (2012) Molecular Cloning: A Laboratory Manual, 4$^{th}$ edition; the series Ausubel et al. eds. (2015) Current Protocols in Molecular Biology; the series Methods in Enzymology (Academic Press, Inc., N.Y.); MacPherson et al. (2015) PCR 1: A Practical Approach (IRL Press at Oxford University Press); MacPherson et al. (1995) PCR 2: A Practical Approach; McPherson et al. (2006) PCR: The Basics (Garland Science); Harlow and Lane eds. (1999) Antibodies, A Laboratory Manual; Greenfield ed. (2014) Antibodies, A Laboratory Manual; Freshney (2010) Culture of Animal Cells: A Manual of Basic Technique, 6$^{th}$ edition; Gait ed. (1984) Oligonucleotide Synthesis; U.S. Pat. No. 4,683,195; Hames and Higgins eds. (1984) Nucleic Acid Hybridization; Anderson (1999) Nucleic Acid Hybridization; Herdewijn ed. (2005) Oligonucleotide Synthesis: Methods and Applications; Hames and Higgins eds. (1984) Transcription and Translation; Buzdin and Lukyanov ed. (2007) Nucleic Acids Hybridization: Modern Applications; Immobilized Cells and Enzymes (IRL Press (1986)); Grandi ed. (2007) *In Vitro* Transcription and Translation Protocols, 2$^{nd}$ edition; Guisan ed. (2006) Immobilization of Enzymes and Cells; Perbal (1988) A Practical Guide to Molecular Cloning, 2$^{nd}$ edition; Miller and Calos eds, (1987)

Gene Transfer Vectors for Mammalian Cells (Cold Spring Harbor Laboratory); Makrides ed. (2003) Gene Transfer and Expression in Mammalian Cells; Mayer and Walker eds. (1987) Immunochemical Methods in Cell and Molecular Biology (Academic Press, London); Lundblad and Macdonald eds. (2010) Handbook of Biochemistry and Molecular Biology, 4th edition; and Herzenberg et al. eds (1996) Weir's Handbook of Experimental Immunology, 5th edition; and the more recent editions each thereof available at the time of filing.

**[0061]** All numerical designations, e.g., pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied ( + ) or ( - ) by increments of 1.0 or 0.1, as appropriate, or alternatively by a variation of +/- 15 %, or alternatively 10%, or alternatively 5%, or alternatively 2%. It is to be understood, although not always explicitly stated, that all numerical designations are preceded by the term "about". It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

**[0062]** It is to be inferred without explicit recitation and unless otherwise intended, that when the present technology relates to a polypeptide, protein, polynucleotide or antibody, an equivalent or a biologically equivalent of such is intended within the scope of the present technology.

***Definitions***

**[0063]** As used in the specification and claims, the singular form "a", "an", and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

**[0064]** As used herein, the term "animal" refers to living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term "mammal" includes both human and non-human mammals.

**[0065]** The terms "subject," "host," "individual," and "patient" are as used interchangeably herein to refer to human and veterinary subjects, for example, humans, animals, non-human primates, dogs, cats, sheep, mice, horses, and cows. In some embodiments, the subject is a human.

**[0066]** As used herein, the term "antibody" collectively refers to immunoglobulins or immunoglobulin-like molecules including by way of example and without limitation, IgA,

IgD, IgE, IgG and IgM, combinations thereof, and similar molecules produced during an immune response in any vertebrate, for example, in mammals such as humans, goats, rabbits and mice, as well as non-mammalian species, such as shark immunoglobulins. Unless specifically noted otherwise, the term "antibody" includes intact immunoglobulins and "antibody fragments" or "antigen binding fragments" that specifically bind to a molecule of interest (or a group of highly similar molecules of interest) to the substantial exclusion of binding to other molecules (for example, antibodies and antibody fragments that have a binding constant for the molecule of interest that is at least $10^3$ $M^{-1}$ greater, at least $10^4$ $M^{-1}$ greater or at least $10^5$ $M^{-1}$ greater than a binding constant for other molecules in a biological sample). The term "antibody" also includes genetically engineered forms such as chimeric antibodies (for example, murine or humanized non-primate antibodies), heteroconjugate antibodies (such as, bispecific antibodies). See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, Ill.); Owen et al., *Kuby Immunology*, 7th Ed., W.H. Freeman & Co., 2013; Murphy, *Janeway's Immunobiology*, 8th Ed., Garland Science, 2014; Male et al., *Immunology* (Roitt), 8th Ed., Saunders, 2012; Parham, *The Immune System*, 4th Ed., Garland Science, 2014.

**[0067]** As used herein, the term "monoclonal antibody" refers to an antibody produced by a single clone of B-lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. Monoclonal antibodies include humanized monoclonal antibodies.

**[0068]** In terms of antibody structure, an immunoglobulin has heavy (H) chains and light (L) chains interconnected by disulfide bonds. There are two types of light chain, lambda ($\lambda$) and kappa ($\kappa$). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Each heavy and light chain contains a constant region and a variable region, (the regions are also known as "domains"). In combination, the heavy and the light chain variable regions specifically bind the antigen. Light and heavy chain variable regions contain a "framework" region interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs". The extent of the framework region and CDRs have been defined (*see*, Kabat et al., *Sequences of Proteins of Immunological Interest*, U.S. Department of Health

and Human Services, 1991, which is hereby incorporated by reference). The Kabat database is now maintained online. The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, largely adopts a β-sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β-sheet structure. Thus, framework regions act to form a scaffold that provides for positioning the CDRs in correct orientation by inter-chain, non-covalent interactions.

**[0069]** The CDRs are primarily responsible for binding to an epitope of an antigen. The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are also typically identified by the chain in which the particular CDR is located (heavy chain regions labeled CDHR and light chain regions labeled CDLR). Thus, a CDHR3 is the CDR3 from the variable domain of the heavy chain of the antibody in which it is found, whereas a CDLR1 is the CDR1 from the variable domain of the light chain of the antibody in which it is found. For example, a TNT antibody will have a specific $V_H$ region and the $V_L$ region sequence unique to the TNT relevant antigen, and thus specific CDR sequences. Antibodies with different specificities (i.e., different combining sites for different antigens) have different CDRs. Although it is the CDRs that vary from antibody to antibody, only a limited number of amino acid positions within the CDRs are directly involved in antigen binding. These positions within the CDRs are called specificity determining residues (SDRs).

**[0070]** As used herein, the term "antigen" refers to a compound, composition, or substance that may be specifically bound by the products of specific humoral or cellular immunity, such as an antibody molecule or T-cell receptor. Antigens can be any type of molecule including, for example, haptens, simple intermediary metabolites, sugars (e.g., oligosaccharides), lipids, and hormones as well as macromolecules such as complex carbohydrates (e.g., polysaccharides), phospholipids, and proteins. Common categories of antigens include, but are not limited to, viral antigens, bacterial antigens, fungal antigens, protozoa and other parasitic antigens, tumor antigens, antigens involved in autoimmune disease, allergy and graft rejection, toxins, and other miscellaneous antigens.

**[0071]** As used herein, the term "antigen binding domain" refers to any protein or polypeptide domain that can specifically bind to an antigen target.

**[0072]** As used herein, the term "autologous," in reference to cells refers to cells that are isolated and infused back into the same subject (recipient or host). "Allogeneic" refers to non-autologous cells.

**[0073]** As used herein, the term "B cell," refers to a type of lymphocyte in the humoral immunity of the adaptive immune system. B cells principally function to make antibodies, serve as antigen presenting cells, release cytokines, and develop memory B cells after activation by antigen interaction. B cells are distinguished from other lymphocytes, such as T cells, by the presence of a B-cell receptor on the cell surface. B cells may either be isolated or obtained from a commercially available source. Non-limiting examples of commercially available B cell lines include lines AHH-1 (ATCC® CRL-8146™), BC-1 (ATCC® CRL-2230™), BC-2 (ATCC® CRL-2231™), BC-3 (ATCC® CRL-2277™), CA46 (ATCC® CRL-1648™), DG-75 [D.G.-75] (ATCC® CRL-2625™), DS-1 (ATCC® CRL-11102™), EB-3 [EB3] (ATCC® CCL-85™), Z-138 (ATCC #CRL-3001), DB (ATCC CRL-2289), Toledo (ATCC CRL-2631), Pfiffer (ATCC CRL-2632), SR (ATCC CRL-2262), JM-1 (ATCC CRL-10421), NFS-5 C-1 (ATCC CRL-1693); NFS-70 C10 (ATCC CRL-1694), NFS-25 C-3 (ATCC CRL-1695), AND SUP-B15 (ATCC CRL-1929). Further examples include but are not limited to cell lines deived from anaplastic and large cell lymphomas, e.g., DEL, DL-40, FE-PD, JB6, Karpas 299, Ki-JK, Mac-2A Ply1, SR-786, SU-DHL-1, -2, -4, -5,-6,-7,-8,-9,-10, and -16, DOHH-2, NU-DHL-1, U-937, Granda 519, USC-DHL-1, RL; Hodgkin's lymphomas, e.g., DEV, HD-70, HDLM-2, HD-MyZ, HKB-1, KM-H2, L 428, L 540, L1236, SBH-1, SUP-HD1, SU/RH-HD-1. Non-limiting exemplary sources for such commercially available cell lines include the American Type Culture Collection, or ATCC, (http://www.atcc.org/) and the German Collection of Microorganisms and Cell Cultures (https://www.dsmz.de/).

**[0074]** As used herein, a "cancer" is a disease state characterized by the presence in a subject of cells demonstrating abnormal uncontrolled replication and in some aspects, the term may be used interchangably with the term "tumor." The term "cancer or tumor antigen" referes to an antigen known to be associated and expressed on the surface with a cancer cell or tumor cell or tissue, and the term "cancer or tumor targeting antibody" refers to an antibody that targets such an antigen.

**[0075]** The term "chimeric antigen receptor" (CAR), as used herein, refers to a fused protein comprising an extracellular domain capable of binding to an antigen, a

transmembrane domain derived from a polypeptide different from a polypeptide from which the extracellular domain is derived, and at least one intracellular domain. The "chimeric antigen receptor (CAR)" is sometimes called a "chimeric receptor", a "T-body", or a "chimeric immune receptor (CIR)." The "extracellular domain capable of binding to an antigen" means any oligopeptide or polypeptide that can bind to a certain antigen. The "intracellular domain" or "intracellular signaling domain" means any oligopeptide or polypeptide known to function as a domain that transmits a signal to cause activation or inhibition of a biological process in a cell. In certain embodiments, the intracellular domain may comprise, alternatively consist essentially of, or yet further comprise one or more costimulatory signaling domains in addition to the primary signaling domain. The "transmembrane domain" means any oligopeptide or polypeptide known to span the cell membrane and that can function to link the extracellular and signaling domains. A chimeric antigen receptor may optionally comprise a "hinge domain" which serves as a linker between the extracellular and transmembrane domains. Non limiting examples of such domains are provided herein, e.g.:

Hinge domain: IgG1 heavy chain hinge coding sequence:

CTCGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCG

Additional non-limiting example includes an IgG4 hinge region, IgD and CD8 domains, as known in the art.

Transmembrane domain: CD28 transmembrane region coding sequence:

TTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAA
CAGTGGCCTTTATTATTTTCTGGGTG

Intracellular domain: 4-1BB co-stimulatory signaling region coding sequence:

AAACGGGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATGAGACC
AGTACAAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAG
AAGAAGGAGGATGTGAACTG

Intracellular domain: CD28 co-stimulatory signaling region coding sequence:

AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCG
CCGCCCCGGGCCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTT
CGCAGCCTATCGCTCC

Intracellular domain: CD3 zeta signaling region coding sequence:

AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGAA
CCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGG
ACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAA
CCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCT
ACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGG
CCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACAT
GCAGGCCCTGCCCCCTCGCTAA

**[0076]** Further embodiments of each exemplary domain component include other proteins that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid sequence identity, preferably 90% sequence identity, more preferably at least 95% sequence identity with the proteins encoded by the above disclosed nucleic acid sequences. Further, non limiting examples of such domains are provided herein.

**[0077]** As used herein, the term "CD8 α hinge domain" refers to a specific protein fragment associated with this name and any other molecules that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid sequence identity, preferably 90% sequence identity, more preferably at least 95% sequence identity with the CD8 α hinge domain sequence as shown herein. The example sequences of CD8 α hinge domain for human, mouse, and other species are provided in Pinto, R.D. et al. (2006) Vet. Immunol. Immunopathol. 110:169-177. The sequences associated with the CD8 α hinge domain are provided in Pinto, R.D. et al. (2006) Vet. Immunol. Immunopathol. 110:169-177. Non-limiting examples of such include:
Human CD8 alpha hinge domain:
PAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIY

Mouse CD8 alpha hinge domain:
KVNSTTTKPVLRTPSPVHPTGTSQPQRPEDCRPRGSVKGTGLDFACDIY

Cat CD8 alpha hinge domain:
PVKPTTTPAPRPPTQAPITTSQRVSLRPGTCQPSAGSTVEASGLDLSCDIY

**[0078]** As used herein, the term "CD8 α transmembrane domain" refers to a specific protein fragment associated with this name and any other molecules that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid

sequence identity, preferably 90% sequence identity, more preferably at least 95% sequence identity with the CD8 α transmembrane domain sequence as shown herein. The fragment sequences associated with the amino acid positions 183 to 203 of the human T-cell surface glycoprotein CD8 alpha chain (GenBank Accession No: NP_001759.3), or the amino acid positions 197 to 217 of the mouse T-cell surface glycoprotein CD8 alpha chain (GenBank Accession No: NP_001074579.1), and the amino acid positions 190 to 210 of the rat T-cell surface glycoprotein CD8 alpha chain(GenBank Accession No: NP_ 113726.1) provide additional example sequences of the CD8 α transmembrane domain. The sequences associated with each of the listed accession numbers are provided as follows:

Human CD8 alpha transmembrane domain: IYIWAPLAGTCGVLLLSLVIT

Mouse CD8 alpha transmembrane domain: IWAPLAGICVALLLSLIITLI

Rat CD8 alpha transmembrane domain: IWAPLAGICAVLLLSLVITLI

**[0079]** As used herein, the term "CD28 transmembrane domain" refers to a specific protein fragment associated with this name and any other molecules that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid sequence identity, at least 90% sequence identity, or alternatively at least 95% sequence identity with the CD28 transmembrane domain sequence as shown herein. The fragment sequences associated with the GenBank Accession Nos: XM_006712862.2 and XM_009444056.1 provide additional, non-limiting, example sequences of the CD28 transmembrane domain. The sequences associated with each of the listed accession numbers are provided herein.

**[0080]** As used herein, the term "4-1BB costimulatory signaling region" refers to a specific protein fragment associated with this name and any other molecules that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid sequence identity, preferably 90% sequence identity, more preferably at least 95% sequence identity with the 4-1BB costimulatory signaling region sequence as shown herein. Non-limiting example sequences of the 4-1BB costimulatory signaling region are provided in U.S. Publication 20130266551A1 (filed as U.S. App. No. 13/826,258), such as the exemplary sequence provided below:

4-1BB costimulatory signaling region:
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

**[0081]** As used herein, the term "CD28 costimulatory signaling region" refers to a specific protein fragment associated with this name and any other molecules that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid sequence identity, preferably 90% sequence identity, more preferably at least 95% sequence identity with the CD28 costimulatory signaling region sequence shown herein. The example sequences CD28 costimulatory signaling domain are provided in U.S. Patent No. 5,686,281; Geiger, T.L. et al., Blood 98: 2364-2371 (2001); Hombach, A. et al., J Immunol 167: 6123-6131 (2001); Maher, J. et al. Nat Biotechnol 20: 70-75 (2002); Haynes, N.M. et al., J Immunol 169: 5780-5786 (2002); Haynes, N.M. et al., Blood 100: 3155-3163 (2002). Non-limiting examples include residues 114-220 of the below CD28 Sequence: MLRLLLALNL FPSIQVTGNK ILVKQSPMLV AYDNAVNLSC KYSYNLFSRE FRASLHKGLDSAVEVCVVYG NYSQQLQVYS KTGFNCDGKL GNESVTFYLQ NLYVNQTDIY FCKIEVMYPPPYLDNEKSNG TIIHVKGKHL CPSPLFPGPS KPFWVLVVVG GVLACYSLLVTVAFIIFWVR SKRSRLLHSD YMNMTPRRPG PTRKHYQPYA PPRDFAAYRS, and equivalents thereof.

**[0082]** As used herein, the term "ICOS costimulatory signaling region" refers to a specific protein fragment associated with this name and any other molecules that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid sequence identity, preferably 90% sequence identity, more preferably at least 95% sequence identity with the ICOS costimulatory signaling region sequence as shown herein. Non-limiting example sequences of the ICOS costimulatory signaling region are provided in U.S. Publication 2015/0017141A1 the exemplary polynucleotide sequence provided below.

ICOS costimulatory signaling region coding sequence:

ACAAAAAAGA AGTATTCATC CAGTGTGCAC GACCCTAACG GTGAATACAT GTTCATGAGA GCAGTGAACA CAGCCAAAAA ATCCAGACTC ACAGATGTGA CCCTA

**[0083]** As used herein, the term "OX40 costimulatory signaling region" refers to a specific protein fragment associated with this name and any other molecules that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid sequence identity, or alternativley 90% sequence identity, or alternatively at least 95% sequence identity with the OX40 costimulatory signaling region sequence as shown

herein. Non-limiting example sequences of the OX40 costimulatory signaling region are disclosed in U.S. Publication 2012/20148552A1, and include the exemplary sequence provided below.

OX40 costimulatory signaling region coding sequence:

AGGGACCAG AGGCTGCCCC CCGATGCCCA CAAGCCCCCT GGGGGAGGCA GTTTCCGGAC CCCCATCCAA GAGGAGCAGG CCGACGCCCA CTCCACCCTG GCCAAGATC, and equivalents thereof.

**[0084]** As used herein, the term "CD28 costimulatory signaling region" refers to a specific protein fragment associated with this name and any other molecules that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid sequence identity, or alternatively 90% sequence identity, or alternatively at least 95% sequence identity with the CD28 costimulatory signaling region sequence shown herein. The example sequences CD28 costimulatory signaling domain are provided in U.S. Patent No. 5,686,281; Geiger, T.L. et al. (2001) Blood 98: 2364-2371; Hombach, A. et al. (2001) J Immunol 167: 6123-6131; Maher, J. et al. (2002) Nat Biotechnol 20: 70-75; Haynes, N.M. et al. (2002) J Immunol 169: 5780-5786 (2002); Haynes, N.M. et al. (2002) Blood 100: 3155-3163. Non-limiting examples include residues 114-220 of the below and the sequence encoded:
CD28 Sequence: MLRLLLALNL FPSIQVTGNK ILVKQSPMLV AYDNAVNLSC KYSYNLFSRE FRASLHKGLDSAVEVCVVYG NYSQQLQVYS KTGFNCDGKL GNESVTFYLQ NLYVNQTDIY FCKIEVMYPPPYLDNEKSNG TIIHVKGKHL CPSPLFPGPS KPFWVLVVVG GVLACYSLLVTVAFIIFWVR SKRSRLLHSD YMNMTPRRPG PTRKHYQPYA PPRDFAAYRS, and equivalents thereof.

**[0085]** As used herein, the term "CD3 zeta signaling domain" refers to a specific protein fragment associated with this name and any other molecules that have analogous biological function that share at least 70%, or alternatively at least 80% amino acid sequence identity, preferably 90% sequence identity, more preferably at least 95% sequence identity with the CD3 zeta signaling domain sequence as shown herein. Non-limiting example sequences of the CD3 zeta signaling domain are provided in U.S. Application No. 13/826,258, e.g.:
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD ALHMQALPPR

**[0086]** As used herein, the term NKG2D refers to an activating receptor that has recently generated considerable interest. A number of NKG2D target ligands have been identified. The most intriguing of these are a pair of closely related proteins called MICA and MICB (major histocompatibility complex (MHC) class I chain-related).

**[0087]** As used herein, the term ULBP refers to a member of th eUL16-binding protein family.

**[0088]** A "composition" typically intends a combination of the active agent, *e.g.*, compound or composition, and a naturally-occurring or non-naturally-occurring carrier, inert (for example, a detectable agent or label) or active, such as an adjuvant, diluent, binder, stabilizer, buffers, salts, lipophilic solvents, preservative, adjuvant or the like and include pharmaceutically acceptable carriers. Carriers also include pharmaceutical excipients and additives proteins, peptides, amino acids, lipids, and carbohydrates (*e.g.*, sugars, including monosaccharides, di-, tri-, tetra-oligosaccharides, and oligosaccharides; derivatized sugars such as alditols, aldonic acids, esterified sugars and the like; and polysaccharides or sugar polymers), which can be present singly or in combination, comprising alone or in combination 1-99.99% by weight or volume. Exemplary protein excipients include serum albumin such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, and the like. Representative amino acid/antibody components, which can also function in a buffering capacity, include alanine, arginine, glycine, arginine, betaine, histidine, glutamic acid, aspartic acid, cysteine, lysine, leucine, isoleucine, valine, methionine, phenylalanine, aspartame, and the like. Carbohydrate excipients are also intended within the scope of this technology, examples of which include but are not limited to monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol) and myoinositol.

**[0089]** As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but do not exclude others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination for the intended use. For example, a composition consisting essentially of the elements as defined herein would not

exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions disclosed herein. Aspects defined by each of these transition terms are within the scope of the present disclosure.

**[0090]** The term "consensus sequence" as used herein refers to an amino acid or nucleic acid sequence that is determined by aligning a series of multiple sequences and that defines an idealized sequence that represents the predominant choice of amino acid or base at each corresponding position of the multiple sequences. Depending on the sequences of the series of multiple sequences, the consensus sequence for the series can differ from each of the sequences by zero, one, a few, or more substitutions. Also, depending on the sequences of the series of multiple sequences, more than one consensus sequence may be determined for the series. The generation of consensus sequences has been subjected to intensive mathematical analysis. Various software programs can be used to determine a consensus sequence.

**[0091]** As used herein, the term "CRISPR" refers to a technique of sequence specific genetic manipulation relying on the clustered regularly interspaced short palindromic repeats pathway. CRISPR can be used to perform gene editing and/or gene regulation, as well as to simply target proteins to a specific genomic location. Gene editing refers to a type of genetic engineering in which the nucleotide sequence of a target polynucleotide is changed through introduction of deletions, insertions, or base substitutions to the polynucleotide sequence. In some aspects, CRISPR-mediated gene editing utilizes the pathways of nonhomologous end-joining (NHEJ) or homologous recombination to perform the edits. Gene regulation refers to increasing or decreasing the production of specific gene products such as protein or RNA.

**[0092]** The term "gRNA" or "guide RNA" as used herein refers to the guide RNA sequences used to target specific genes for correction employing the CRISPR technique. Techniques of designing gRNAs and donor therapeutic polynucleotides for target specificity are well known in the art. For example, Doench, J., et al. Nature biotechnology 2014; 32(12):1262-7, Mohr, S. et al. (2016) FEBS Journal 283: 3232-38, and Graham, D., et al. Genome Biol. 2015; 16: 260. gRNA comprises or alternatively consists essentially of, or yet further consists of a fusion polynucleotide comprising

CRISPR RNA (crRNA) and trans-activating CRIPSPR RNA (tracrRNA); or a polynucleotide comprising CRISPR RNA (crRNA) and trans-activating CRIPSPR RNA (tracrRNA). In some aspects, a gRNA is synthetic (Kelley, M. et al. (2016) J of Biotechnology 233 (2016) 74-83). As used herehin, a biological equivalent of a gRNA includes but is not limited to polynucleotides or targeting molecules that can guide a Cas9 or equivalent thereof to a specific nucleotide sequence such as a specific region of a cell's genome.

**[0093]** "Cytoreductive therapy," as used herein, includes but is not limited to chemotherapy, cryotherapy, and radiation therapy. Agents that act to reduce cellular proliferation are known in the art and widely used. Chemotherapy drugs that kill cancer cells only when they are dividing are termed cell-cycle specific. These drugs include agents that act in S-phase, including topoisomerase inhibitors and anti-metabolites.

**[0094]** Toposiomerase inhibitors are drugs that interfere with the action of topoisomerase enzymes (topoisomerase I and II). During the process of chemo treatments, topoisomerase enzymes control the manipulation of the structure of DNA necessary for replication, and are thus cell cycle specific. Examples of topoisomerase I inhibitors include the camptothecan analogs listed above, irinotecan and topotecan. Examples of topoisomerase II inhibitors include amsacrine, etoposide, etoposide phosphate, and teniposide.

**[0095]** Antimetabolites are usually analogs of normal metabolic substrates, often interfering with processes involved in chromosomal replication. They attack cells at very specific phases in the cycle. Antimetabolites include folic acid antagonists, e.g., methotrexate; pyrimidine antagonist, e.g., 5-fluorouracil, foxuridine, cytarabine, capecitabine, and gemcitabine; purine antagonist, e.g., 6-mercaptopurine and 6-thioguanine; adenosine deaminase inhibitor, e.g., cladribine, fludarabine, nelarabine and pentostatin; and the like.

**[0096]** Plant alkaloids are derived from certain types of plants. The vinca alkaloids are made from the periwinkle plant (*Catharanthus rosea*). The taxanes are made from the bark of the Pacific Yew tree (taxus). The vinca alkaloids and taxanes are also known as antimicrotubule agents. The podophyllotoxins are derived from the May apple plant. Camptothecan analogs are derived from the Asian "Happy Tree" (*Camptotheca acuminata*). Podophyllotoxins and camptothecan analogs are also classified as topoisomerase inhibitors. The plant alkaloids are generally cell-cycle specific.

**[0097]** Examples of these agents include vinca alkaloids, e.g., vincristine, vinblastine and vinorelbine; taxanes, e.g., paclitaxel and docetaxel; podophyllotoxins, e.g., etoposide and tenisopide; and camptothecan analogs, e.g., irinotecan and topotecan.

**[0098]** Cryotherapy includes, but is not limited to, therapies involving decreasing the temperature, for example, hypothermic therapy.

**[0099]** Radiation therapy includes, but is not limited to, exposure to radiation, e.g., ionizing radiation, UV radiation, as known in the art. Exemplary dosages include, but are not limited to, a dose of ionizing radiation at a range from at least about 2 Gy to not more than about 10 Gy and/or a dose of ultraviolet radiation at a range from at least about 5 $J/m^2$ to not more than about 50 $J/m^2$, usually about 10 $J/m^2$.

**[0100]** As used herein, the term "detectable marker" refers to at least one marker capable of directly or indirectly, producing a detectable signal. A non-exhaustive list of this marker includes enzymes which produce a detectable signal, for example by colorimetry, fluorescence, luminescence, such as horseradish peroxidase, alkaline phosphatase, β-galactosidase, glucose-6-phosphate dehydrogenase, chromophores such as fluorescent, luminescent dyes, groups with electron density detected by electron microscopy or by their electrical property such as conductivity, amperometry, voltammetry, impedance, detectable groups, for example whose molecules are of sufficient size to induce detectable modifications in their physical and/or chemical properties, such detection may be accomplished by optical methods such as diffraction, surface plasmon resonance, surface variation , the contact angle change or physical methods such as atomic force spectroscopy, tunnel effect, or radioactive molecules such as $^{32}P$, $^{35}S$ or $^{125}I$.

**[0101]** An "effective amount" or "efficacious amount" refers to the amount of an agent, or combined amounts of two or more agents, that, when administered for the treatment of a mammal or other subject, is sufficient to effect such treatment for the disease. The "effective amount" will vary depending on the agent(s), the disease and its severity and the age, weight, etc., of the subject to be treated.

**[0102]** The term "encode" as it is applied to nucleic acid sequences refers to a polynucleotide which is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, can be transcribed and/or translated to produce the mRNA for the polypeptide and/or a fragment thereof.

The antisense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

**[0103]** As used herein, the term "enhancer", as used herein, denotes sequence elements that augment, improve or ameliorate transcription of a nucleic acid sequence irrespective of its location and orientation in relation to the nucleic acid sequence to be expressed. An enhancer may enhance transcription from a single promoter or simultaneously from more than one promoter. As long as this functionality of improving transcription is retained or substantially retained (e.g., at least 70%, at least 80%, at least 90% or at least 95% of wild-type activity, that is, activity of a full-length sequence), any truncated, mutated or otherwise modified variants of a wild-type enhancer sequence are also within the above definition.

**[0104]** In one aspect, the term "equivalent" or "biological equivalent" of an antibody means the ability of the antibody to selectively bind its epitope protein or fragment thereof as measured by ELISA or other suitable methods. Biologically equivalent antibodies include, but are not limited to, those antibodies, peptides, antibody fragments, antibody variant, antibody derivative and antibody mimetics that bind to the same epitope as the reference antibody.

**[0105]** It is to be inferred without explicit recitation and unless otherwise intended, that when the present disclosure relates to a polypeptide, protein, polynucleotide or antibody, an equivalent or a biologically equivalent of such is intended within the scope of this disclosure. As used herein, the term "biological equivalent thereof" is intended to be synonymous with "equivalent thereof" when referring to a reference protein, antibody, polypeptide or nucleic acid, intends those having minimal homology while still maintaining desired structure or functionality. Unless specifically recited herein, it is contemplated that any polynucleotide, polypeptide or protein mentioned herein also includes equivalents thereof. For example, an equivalent intends at least about 70% homology or identity, or at least 80 % homology or identity and alternatively, or at least about 85 %, or alternatively at least about 90 %, or alternatively at least about 95 %, or alternatively 98 % percent homology or identity and exhibits substantially equivalent biological activity to the reference protein, polypeptide or nucleic acid. Alternatively, when referring to polynucleotides, an equivalent thereof is a polynucleotide that hybridizes under stringent conditions to the reference polynucleotide or its complement.

**[0106]** A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) having a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. The alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in Current Protocols in Molecular Biology (Ausubel et al., eds. 1987) Supplement 30, section 7.7.18, Table 7.7.1. Preferably, default parameters are used for alignment. A preferred alignment program is BLAST, using default parameters. In particular, preferred programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the following Internet address: ncbi.nlm.nih.gov/cgi-bin/BLAST.

**[0107]** As used herein, the term "expression" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell. The expression level of a gene may be determined by measuring the amount of mRNA or protein in a cell or tissue sample. In one aspect, the expression level of a gene from one sample may be directly compared to the expression level of that gene from a control or reference sample. In another aspect, the expression level of a gene from one sample may be directly compared to the expression level of that gene from the same sample following administration of a compound.

**[0108]** The phrase "first line" or "second line" or "third line" refers to the order of treatment received by a patient. First line therapy regimens are treatments given first, whereas second or third line therapy are given after the first line therapy or after the second line therapy, respectively. The National Cancer Institute defines first line therapy as "the first treatment for a disease or condition. In patients with cancer, primary treatment can be surgery, chemotherapy, radiation therapy, or a combination of these therapies. First line therapy is also referred to those skilled in the art as "primary therapy and primary treatment." See National Cancer Institute website at www.cancer.gov, last visited on May

1, 2008. Typically, a patient is given a subsequent chemotherapy regimen because the patient did not show a positive clinical or sub-clinical response to the first line therapy or the first line therapy has stopped.

**[0109]** As used herein, "homology" or "identical", percent "identity" or "similarity", when used in the context of two or more nucleic acids or polypeptide sequences, refers to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, e.g., at least 60% identity, preferably at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region (e.g., nucleotide sequence encoding an antibody described herein or amino acid sequence of an antibody described herein). Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. The alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in Current Protocols in Molecular Biology (Ausubel et al., eds. 1987) Supplement 30, section 7.7.18, Table 7.7.1. Preferably, default parameters are used for alignment. A preferred alignment program is BLAST, using default parameters. In particular, preferred programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the following Internet address: ncbi.nlm.nih.gov/cgi-bin/BLAST. The terms "homology" or "identical", percent "identity" or "similarity" also refer to, or can be applied to, the complement of a test sequence. The terms also include sequences that have deletions and/or additions, as well as those that have substitutions. As described herein, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is at least 50-100 amino acids or nucleotides in length. An "unrelated" or "non-homologous" sequence shares less than 40% identity, or alternatively less than 25% identity, with one of the sequences disclosed herein.

**[0110]** "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

**[0111]** Examples of stringent hybridization conditions include: incubation temperatures of about 25°C to about 37°C; hybridization buffer concentrations of about 6x SSC to about 10x SSC; formamide concentrations of about 0% to about 25%; and wash solutions from about 4x SSC to about 8x SSC. Examples of moderate hybridization conditions include: incubation temperatures of about 40°C to about 50°C; buffer concentrations of about 9x SSC to about 2x SSC; formamide concentrations of about 30% to about 50%; and wash solutions of about 5x SSC to about 2x SSC. Examples of high stringency conditions include: incubation temperatures of about 55°C to about 68°C; buffer concentrations of about 1x SSC to about 0.1x SSC; formamide concentrations of about 55% to about 75%; and wash solutions of about 1x SSC, 0.1x SSC, or deionized water. In general, hybridization incubation times are from 5 minutes to 24 hours, with 1, 2, or more washing steps, and wash incubation times are about 1, 2, or 15 minutes. SSC is 0.15 M NaCl and 15 mM citrate buffer. It is understood that equivalents of SSC using other buffer systems can be employed.

**[0112]** The term "isolated" as used herein refers to molecules or biologicals or cellular materials being substantially free from other materials. In one aspect, the term "isolated" refers to nucleic acid, such as DNA or RNA, or protein or polypeptide (e.g., an antibody or derivative thereof), or cell or cellular organelle, or tissue or organ, separated from other DNAs or RNAs, or proteins or polypeptides, or cells or cellular organelles, or tissues or organs, respectively, that are present in the natural source. The term "isolated" also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and

would not be found in the natural state. The term "isolated" is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides. The term "isolated" is also used herein to refer to cells or tissues that are isolated from other cells or tissues and is meant to encompass both cultured and engineered cells or tissues.

**[0113]** As used herein, the term "isolated cell" generally refers to a cell that is substantially separated from other cells of a tissue.

**[0114]** "Immune cells" includes, e.g., white blood cells (leukocytes) which are derived from hematopoietic stem cells (HSC) produced in the bone marrow, lymphocytes (T cells, B cells, natural killer (NK) cells) and myeloid-derived cells (neutrophil, eosinophil, basophil, monocyte, macrophage, dendritic cells).

**[0115]** As used herein the term "linker sequence" relates to any amino acid sequence comprising from 1 to 10, or alternatively, 8 amino acids, or alternatively 6 amino acids, or alternatively 5 amino acids that may be repeated from 1 to 10, or alternatively to about 8, or alternatively to about 6, or alternatively about 5, or 4 or alternatively 3, or alternatively 2 times. For example, the linker may comprise up to 15 amino acid residues consisting of a pentapeptide repeated three times. In one aspect, the linker sequence is a (Glycine4Serine)3 flexible polypeptide linker comprising three copies of gly-gly-gly-gly-ser.

**[0116]** A "normal cell corresponding to the tumor tissue type" refers to a normal cell from a same tissue type as the tumor tissue. A non-limiting example is a normal lung cell from a patient having lung tumor, or a normal colon cell from a patient having colon tumor.

**[0117]** As used herein, the term "T cell," refers to a type of lymphocyte that matures in the thymus. T cells play an important role in cell-mediated immunity and are distinguished from other lymphocytes, such as B cells, by the presence of a T-cell receptor on the cell surface. T-cells may either be isolated or obtained from a commercially available source. "T cell" includes all types of immune cells expressing CD3 including T-helper cells (CD4+ cells), cytotoxic T-cells (CD8+ cells), natural killer T-cells, T-regulatory cells (Treg) and gamma-delta T cells. A "cytotoxic cell" includes CD8+ T cells, natural-killer (NK) cells, and neutrophils, which cells are capable of mediating cytotoxicity responses. Non-limiting examples of commercially available T-cell lines include lines BCL2 (AAA) Jurkat (ATCC® CRL-2902™), BCL2 (S70A) Jurkat (ATCC® CRL-2900™), BCL2 (S87A)

Jurkat (ATCC® CRL-2901™), BCL2 Jurkat (ATCC® CRL-2899™), Neo Jurkat (ATCC® CRL-2898™), TALL-104 cytotoxic human T cell line (ATCC # CRL-11386). Further examples include but are not limited to mature T-cell lines, *e.g.*, such as Deglis, EBT-8, HPB-MLp-W, HUT 78, HUT 102, Karpas 384, Ki 225, My-La, Se-Ax, SKW-3, SMZ-1 and T34; and immature T- cell lines, e.g., ALL-SIL, Be13, CCRF-CEM, CML-T1, DND-41, DU.528, EU-9, HD-Mar, HPB-ALL, H-SB2, HT-1, JK-T1, Jurkat, Karpas 45, KE-37, KOPT-K1, K-T1, L-KAW, Loucy, MAT, MOLT-1, MOLT 3, MOLT-4, MOLT 13, MOLT-16, MT-1, MT-ALL, P12/Ichikawa, Peer, PER0117, PER-255, PF-382, PFI-285, RPMI-8402, ST-4, SUP-T1 to T14, TALL-1, TALL-101, TALL-103/2, TALL-104, TALL-105, TALL-106, TALL-107, TALL-197, TK-6, TLBR-1, -2, -3, and -4, CCRF-HSB-2 (CCL-120.1), J.RT3-T3.5 (ATCC TIB-153), J45.01 (ATCC CRL-1990), J.CaM1.6 (ATCC CRL-2063), RS4;11 (ATCC CRL-1873), CCRF-CEM (ATCC CRM-CCL-119); and cutaneous T-cell lymphoma lines, *e.g.*, HuT78 (ATCC CRM-TIB-161), MJ[G11] (ATCC CRL-8294), HuT102 (ATCC TIB-162). Null leukemia cell lines, including but not limited to REH, NALL-1, KM-3, L92-221, are a another commercially available source of immune cells, as are cell lines derived from other leukemias and lymphomas, such as K562 erythroleukemia, THP-1 monocytic leukemia, U937 lymphoma, HEL erythroleukemia, HL60 leukemia, HMC-1 leukemia, KG-1 leukemia, U266 myeloma. Non-limiting exemplary sources for such commercially available cell lines include the American Type Culture Collection, or ATCC, (http://www.atcc.org/) and the German Collection of Microorganisms and Cell Cultures (https://www.dsmz.de/).

**[0118]** As used herein, the term "NK cell," also known as natural killer cell, refers to a type of lymphocyte that originates in the bone marrow and play a critical role in the innate immune system. NK cells provide rapid immune responses against viral-infected cells, tumor cells or other stressed cell, even in the absence of antibodies and major histocompatibility complex on the cell surfaces. NK cells may either be isolated or obtained from a commercially available source. Non-limiting examples of commercial NK cell lines include lines NK-92 (ATCC® CRL-2407™), NK-92MI (ATCC® CRL-2408™). Further examples include but are not limited to NK lines HANK1, KHYG-1, NKL, NK-YS, NOI-90, and YT. Non-limiting exemplary sources for such commercially available cell lines include the American Type Culture Collection, or ATCC, (http://www.atcc.org/) and the German Collection of Microorganisms and Cell Cultures (https://www.dsmz.de/).

**[0119]** As used herein in reference to a regulatory polynucleotide, the term "operatively linked" refers to an association between the regulatory polynucleotide and the polynucleotide sequence to which it is linked such that, when a specific protein binds to the regulatory polynucleotide, the linked polynucleotide is transcribed.

**[0120]** As used herein, the term "overexpress" with respect to a cell, a tissue, or an organ expresses a protein to an amount that is greater than the amount that is produced in a control cell, a control issue, or an organ. A protein that is overexpressed may be endogenous to the host cell or exogenous to the host cell.

**[0121]** The terms "polynucleotide" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, RNAi, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, any aspect of this technology that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

**[0122]** As used herein, the terms "nucleic acid sequence" and "polynucleotide" are used interchangeably to refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases.

**[0123]** The term "promoter" as used herein refers to any sequence that regulates the expression of a coding sequence, such as a gene. Promoters may be constitutive, inducible, repressible, or tissue-specific, for example. A "promoter" is a control sequence that is a region of a polynucleotide sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. Non-limiting examples of promoters include the EF1alpha promoterand the CMV promoter. The EF1alpha sequence is known in the art (see, e.g., addgene.org/11154/sequences/; ncbi.nlm.nih.gov/nuccore/J04617, each last accessed on March 13, 2019, and Zheng and Baum (2014) Int'l. J. Med. Sci. 11(5):404-408). The CMV promoter sequence is known in the art (see, e.g., snapgene.com/resources/plasmid-files/?set=basic_cloning_vectors&plasmid=CMV_promoter, last accessed on March 13, 2019 and Zheng and Baum (2014), supra.).

**[0124]** The term "protein", "peptide" and "polypeptide" are used interchangeably and in their broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs or peptidomimetics. The subunits may be linked by peptide bonds. In another aspect, the subunit may be linked by other bonds, e.g., ester, ether, etc. A protein or peptide must contain at least two amino acids and no limitation is placed on the maximum number of amino acids which may comprise a protein's or peptide's sequence. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D and L optical isomers, amino acid analogs and peptidomimetics.

**[0125]** As used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified nucleic acid, peptide, protein, biological complexes or other active compound is one that is isolated in whole or in part from proteins or other contaminants. Generally, substantially purified peptides, proteins, biological complexes, or other active compounds for use within the disclosure comprise more than 80% of all macromolecular species present in a preparation prior to admixture or formulation of the peptide, protein, biological complex or other active compound with a pharmaceutical carrier, excipient, buffer, absorption enhancing agent, stabilizer, preservative, adjuvant or other co-ingredient in a complete pharmaceutical formulation for

therapeutic administration. More typically, the peptide, protein, biological complex or other active compound is purified to represent greater than 90%, often greater than 95% of all macromolecular species present in a purified preparation prior to admixture with other formulation ingredients. In other cases, the purified preparation may be essentially homogeneous, wherein other macromolecular species are not detectable by conventional techniques.

**[0126]** As used herein, the term "purification marker" refers to at least one marker useful for purification or identification. A non-exhaustive list of this marker includes His, lacZ, GST, maltose-binding protein, NusA, BCCP, c-myc, CaM, FLAG, GFP, YFP, cherry, thioredoxin, poly(NANP), V5, Snap, HA, chitin-binding protein, Softag 1, Softag 3, Strep, or S-protein. Suitable direct or indirect fluorescence marker comprise FLAG, GFP, YFP, RFP, dTomato, cherry, Cy3, Cy 5, Cy 5.5, Cy 7, DNP, AMCA, Biotin, Digoxigenin, Tamra, Texas Red, rhodamine, Alexa fluors, FITC, TRITC or any other fluorescent dye or hapten.

**[0127]** As used herein, the term "recombinant protein" refers to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein.

**[0128]** As used herein, the term "specific binding" means the contact between an antibody and an antigen with a binding affinity of at least $10^{-6}$ M. In certain aspects, antibodies bind with affinities of at least about $10^{-7}$ M, and preferably $10^{-8}$ M, $10^{-9}$ M, $10^{-10}$ M, $10^{-11}$ M, or $10^{-12}$ M.

**[0129]** A "solid tumor" is an abnormal mass of tissue that usually does not contain cysts or liquid areas. Solid tumors can be benign or malignant, metastatic or non-metastatic. Different types of solid tumors are named for the type of cells that form them. Examples of solid tumors include sarcomas, carcinomas, and lymphomas.

**[0130]** As used herein, the term "suicide gene" is a gene capable of inducing cell apoptosis; non-limiting examples include HSV-TK (Herpes simplex virus thymidine kinase), cytosine deaminase, nitroreductase, carboxylesterase, cytochrome P450 or PNP (Purine nucleoside phosphorylase), truncated EGFR, or inducible caspase ("iCasp"). Suicide genes may function along a variety of pathways, and, in some cases, may be

inducible by an inducing agent such as a small molecule. For example, the iCasp suicide gene comprises portion of a caspase protein operatively linked to a protein optimized to bind to an inducing agent; introduction of the inducing agent into a cell comprising the suicide gene results in the activation of caspase and the subsequent apoptosis of said cell.

**[0131]** The term "transduce" or "transduction" as it is applied to the production of chimeric antigen receptor cells refers to the process whereby a foreign nucleotide sequence is introduced into a cell. In some embodiments, this transduction is done via a vector.

**[0132]** As used herein, "treating" or "treatment" of a disease in a subject refers to (1) preventing the symptoms or disease from occurring in a subject that is predisposed or does not yet display symptoms of the disease; (2) inhibiting the disease or arresting its development; or (3) ameliorating or causing regression of the disease or the symptoms of the disease. As understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. For the purposes of the present technology, beneficial or desired results can include one or more, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of a condition (including a disease), stabilized (*i.e.*, not worsening) state of a condition (including disease), delay or slowing of condition (including disease), progression, amelioration or palliation of the condition (including disease), states and remission (whether partial or total), whether detectable or undetectable. Treatments containing the disclosed compositions and methods can be first line, second line, third line, fourth line, fifth line therapy and are intended to be used as a sole therapy or in combination with other appropriate therapies. In one aspect, the term "treatment" or "treating" excludes prevention or prophylaxis.

**[0133]** As used herein, the term "vector" refers to a nucleic acid construct deigned for transfer between different hosts, including but not limited to a plasmid, a virus, a cosmid, a phage, a BAC, a YAC, etc. In some embodiments, plasmid vectors may be prepared from commercially available vectors. In other embodiments, viral vectors may be produced from baculoviruses, retroviruses, adenoviruses, AAVs, etc. according to techniques known in the art. In one embodiment, the viral vector is a lentiviral vector.

**[0134]** As used herein the term "NKG2D" refers to a transmembrane protein belonging to the CD94/NKG2 family of C-type lectin-like receptors and encoded by the gene *KLRK1* gene, which is located in the NK-gene complex and/or a biological equivalent thereof. Non-limiting exemplary sequences of this protein or the underlying gene may be found

under Gene Cards ID: GC12M011728, HGNC: 18788, Entrez Gene: 22914, Ensembl: ENSG00000213809, OMIM: 611817, and UniProtKB: P26718, which are incorporated by reference herein.

**[0135]** As used herein the terms "BCMA" and "B-cell maturation antigen" are used interchangeably to refer to a protein belonging to the TNF superfamily which recognizes B-cell activating factor (BAFF) and encoded by the *TNFRSF17* gene. Non-limiting exemplary sequences of this protein or the underlying gene may be found under Gene Cards ID: GC16P012058, HGNC: 11913, Entrez Gene: 608, Ensembl: ENSG00000048462, OMIM: 109545, and UniProtKB: Q02223, which are incorporated by reference herein.

**[0136]** As used herein the terms "SLAMF7," "CS1," and "CD319" are used interchangeably to refer to a protein known to be a robust marker ot normal plasma cell and malignant plasma cells in multiple myeloma and encoded by the *SLAMF7* gene. Non-limiting exemplary sequences of this protein or the underlying gene may be found under Gene Cards ID: GC01P160709, HGNC: 21394, Entrez Gene: 57823, Ensembl: ENSG00000026751, OMIM: 606625, and UniProtKB: Q9NQ25, which are incorporated by reference herein.

**[0137]** The sequences associated with each of the above listed GenBank Accession Nos. and references are herein incorporated by reference.

## MODES FOR CARRYING OUT THE DISCLOSURE

**[0138]** Administration of immunoregulatory molecules has been pursued as a cancer therapeutic. However, due to severe side effects associated with systemic administration (Giovarelli, M. et al. (2000) J Immunol. 164:3200-3206; Lasek, W. et al. (2014) Cancer Immunol Immunother. 63:419-435), obtaining high concentrations of these immunoregulatory molecules in relevant tumors in order to achieve an effective immune response has been difficult.

**[0139]** Due to the unprecedented results being recently obtained in B-cell lymphomas and leukemia's using autologous treatment with genetically engineered chimeric antigen receptor (CAR) T-cells (Maude, S.L. et al. (2014) New Engl. J. Med. 371:1507-1517; Porter, D.L. et al. (2011) New Engl. J. Med. 365:725-733), a number of laboratories have begun to apply this approach to solid tumors including ovarian cancer, prostate cancer, and pancreatic tumors. CAR modified T-cells combine the HLA-independent targeting

specificity of a monoclonal antibody with the cytolytic activity, proliferation, and homing properties of activated T-cells, but do not respond to checkpoint suppression. Because of their ability to kill antigen expressing targets directly, CAR T-cells are highly toxic to any antigen positive cells or tissues making it a requirement to construct CARs with highly tumor specific antibodies. To date, CAR modified T-cells to human solid tumors have been constructed against the α-folate receptor, mesothelin, and MUC-CD, PSMA, and other targets but most have some off-target expression of antigen in normal tissues. These constructs have not shown the same exceptional results in patients emphasizing the need for additional studies to identify new targets and methods of CAR T-cell construction that can be used against solid tumors.

**[0140]** Thus, this disclosure provides a chimeric antigen receptor (CAR) comprising a binding domain specific to a cancer or tumor antigen, that in some aspects, is the antigen binding domain of an anti-BCMA antibody, a vector encoding a BsA-CAR cell that targets a tumor or cancer antigen and secretes soluble antibody fragments, and methods and compositions relating to the use and production thereof.

***Chimeric Antigen Receptors and Uses Thereof***

***Components***

**[0141]** The present disclosure provides chimeric antigen receptors (CAR) that bind to a cancer or tumor antigen, the CAR comprising, or consisting essentially of, or consisting of, a cell activation moiety comprising an extracellular, transmembrane, and intracellular domain. The extracellular domain comprises a target-specific binding element otherwise referred to as the antigen binding domain. The intracellular domain or cytoplasmic domain comprises a costimulatory signaling region and a zeta chain portion. The CAR may optionally further comprise a spacer domain of up to 300 amino acids, preferably 10 to 100 amino acids, more preferably 25 to 50 amino acids.

**[0142]** *Spacer Domain.* The CAR may optionally further comprise a spacer domain of up to 300 amino acids, preferably 10 to 100 amino acids, more preferably 25 to 50 amino acids. For example, the spacer may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids. A spacer domain may comprise, for example, a portion of a human Fc domain, a CH3 domain, or the hinge region of any

immunoglobulin, such as IgA, IgD, IgE, IgG, or IgM, or variants thereof. For example, some embodiments may comprise an IgG4 hinge with or without a S228P, L235E, and/or N297Q mutation (according to Kabat numbering). Additional spacers include, but are not limited to, CD4, CD8, and CD28 hinge regions. In on

**[0143]** *Antigen Binding Domain.* In certain aspects, the present disclosure provides a CAR that comprises, or alternatively consists essentially thereof, or yet further consists of an antigen binding domain specific to a cancer or tumor antigen. The antigen binding domains can be from any appropriate species, e.g., murine, human or a humanized sequence.

**[0144]** In some embodiments, the antigen binding domain comprises, or alternatively consists essentially thereof, or yet consists of the antigen binding domain of an anti-BCMA antibody or an antibody that binds a BCMA- relevant antigen. Monoclonal antibodies that specifically bind these antigens are commercially available. The antigen binding domains can be from any appropriate species, e.g., murine, human or a humanized sequence. In one aspect, the antigen binding domain comprises the heavy chain variable region and the light chain variable region of an antibody to B-cell maturation antigen (BCMA) and/or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof. In some embodiments, the antigen binding domain comprises, consists, or consists essentially of a fragment of the target-specific antibody (i.e., an antibody to B-cell maturation antigen (BCMA) and/or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof), for example, an scFv. An scFv region can comprise the variable regions of the heavy ($V_H$) and light chains ($V_L$) of immunoglobulins, connected with a short linker peptide. The linker peptide may be from 1 to 50 amino acids, for instance, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids. In some embodiments, the linker is glycine rich, although it may also contain serine or threonine.

**[0145]** In another aspect of the present disclosure, the antigen binding domain of a cancer or tumor antibody includes one or more of the following characteristics:

(a) the light chain immunoglobulin variable domain sequence comprises one or more CDRs that are at least 80% identical to a CDR of a light chain variable domain of any of the disclosed light chain sequences;

(b) the heavy chain immunoglobulin variable domain sequence comprises one or more CDRs that are at least 80% identical to a CDR of a heavy chain variable domain of

any of the disclosed heavy chain sequences;

(c) the light chain immunoglobulin variable domain sequence is at least 80% identical to a light chain variable domain of any of the disclosed light chain sequences;

(d) the HC immunoglobulin variable domain sequence is at least 80% identical to a heavy chain variable domain of any of the disclosed light chain sequences; and

(e) the antibody binds an epitope that overlaps with an epitope bound by any of the disclosed sequences.

**[0146]** Additional examples of equivalents include peptide having at least 85% , or alternatively at least 90%, or alternatively at least 95%, or alternatively at least 97% amino acid identity to the peptide or a polypeptide that is encoded by a polynucleotide that hybridizes under conditions of high stringency to the complement of a polynucleotide encoding the antigen binding domain, wherein conditions of high stringency comprises incubation temperatures of about 55°C to about 68°C; buffer concentrations of about 1x SSC to about 0.1x SSC; formamide concentrations of about 55% to about 75%; and wash solutions of about 1x SSC, 0.1x SSC, or deionized water.

**[0147]** *Transmembrane Domain.* The transmembrane domain may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions of particular use in this disclosure may be derived from CD8, CD28, CD3, CD45, CD4, CD5, CDS, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD 134, CD137, CD 154, TCR. Alternatively, the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR. A glycine-serine doublet provides a particularly suitable linker.

**[0148]** *Cytoplasmic Domain.* The cytoplasmic domain or intracellular signaling domain of the CAR is responsible for activation of at least one of the traditional effector functions of an immune cell in which a CAR has been placed. The intracellular signaling domain refers to a portion of a protein which transduces the effector function signal and directs the immune cell to perform its specific function. An entire signaling domain or a truncated

portion thereof may be used so long as the truncated portion is sufficient to transduce the effector function signal. Cytoplasmic sequences of the TCR and co-receptors as well as derivatives or variants thereof can function as intracellular signaling domains for use in a CAR. Intracellular signaling domains of particular use in this disclosure may be derived from FcR, TCR, CD3, CDS, CD22, CD79a, CD79b, CD66d. In some embodiments, the signaling domain of the CAR can comprise a CD3 ζ signaling domain.

**[0149]** Since signals generated through the TCR are alone insufficient for full activation of a T cell, a secondary or co-stimulatory signal may also be required. Thus, the intracellular region of a co-stimulatory signaling molecule, including but not limited the intracellular domains of the proteins CD27, CD28, 4- IBB (CD 137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, or a ligand that specifically binds with CD83, may also be included in the cytoplasmic domain of the CAR. For instance, a CAR may comprise one, two, or more co-stimulatory domains, in addition to a signaling domain (e.g., a CD3 ζ signaling domain).

**[0150]** In some embodiments, the cell activation moiety of the chimeric antigen receptor is a T-cell signaling domain comprising, or alternatively consisting essentially of, or yet further consisting of, one or more proteins or fragments thereof selected from the group consisting of CD8 protein, CD28 protein, 4-1BB protein, OX40, CD30, CD40, PD-1, ICOS, LFA-1, CD2, CD7, CD27, LIGHT, NKG2C, B7-H3, and CD3-zeta protein.

**[0151]** In some embodiments, the cell activation moiety of the chimeric antigen receptor is a T-cell signaling domain comprising, or alternatively consisting essentially of, or yet further consisting of, one or more proteins or fragments thereof selected from the group consisting of CD8 protein, CD28 protein, 4-1BB protein, and CD3-zeta protein.

**[0152]** In specific embodiments, the CAR comprises, or alternatively consists essentially thereof, or yet consists of an antigen binding domain of a caner or tumor targeting antibody, a CD8 α hinge domain, a CD8 α transmembrane domain, a costimulatory signaling region, and a CD3 zeta signaling domain. In further embodiments, the costimulatory signaling region comprises either or both a CD28 costimulatory signaling region and a 4-1BB costimulatory signaling region.

**[0153]** In some embodiments, the CAR can further comprise a detectable marker or purification marker. In another aspect, the CARs as described herein are contained in a composition, e.g., a pharmaceutically acceptable carrier for diagnosis or therapy.

**[0154]** *Switch Mechanisms.* In some embodiments, the CAR may also comprise a switch mechanism for controlling expression and/or activation of the CAR. For example, a CAR may comprise, consist, or consist essentially of an extracellular, transmembrane, and intracellular domain, in which the extracellular domain comprises a target-specific binding element that binds a label, binding domain, or tag that is specific for a molecule other than the target antigen that is expressed on or by a target cell. In such embodiments, the specificity of the CAR is provided by a second construct that comprises, consists, or consists essentially of a target antigen binding domain and a domain that is recognized by or binds to the label, binding domain, or tag on the CAR. See, e.g., WO 2013/044225, WO 2016/000304, WO 2015/057834, WO 2015/057852, WO 2016/070061, US 9,233,125, US 2016/0129109. In this way, a T-cell that expresses the CAR can be administered to a subject, but it cannot bind its a target antigen (i.e., BCMA) until the second composition comprising an BCMA-specific binding domain is administered.

**[0155]** CARs of the present disclosure may likewise require multimerization in order to active their function (see, e.g., US 2015/0368342, US 2016/0175359, US 2015/0368360) and/or an exogenous signal, such as a small molecule drug (US 2016/0166613, Yung et al., Science, 2015) in order to elicit a T-cell response.

**[0156]** Furthermore, the disclosed CARs can comprise a "suicide switch" (also referred to as a "suicide gene") to induce cell death of the CAR cells following treatment (Buddee et al., PLoS One, 2013) or to downregulate expression of the CAR following binding to the target antigen (WO 2016/011210). A non-limiting exemplary suicide switch or suicide gene is iCasp.

**[0157]** In some embodiments, the CAR can further comprise a detectable marker or purification marker. In another aspect, the CARs as described herein are contained in a composition, e.g., a pharmaceutically acceptable carrier for diagnosis or therapy.

**[0158]** In certain embodiments, the antigen binding domain of the tumor targeting antibody of the CAR comprises, or alternatively consists essentially thereof, or further consists of a heavy chain variable region and a light chain variable region that are optionally

linked by a linker peptide. In some embodiments, the heavy and/or light chain variable region comprises, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to any one of B-cell maturation antigen (BCMA) and/or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof. In one aspect, the tumor targeting antibody targets BCMA. In certain embodiments, the CAR further comprises, or alternatively further consists essentially of, or yet further consists of, a linker polypeptide located between the heavy chain variable region and the light chain variable region. In certain embodiments, the linker is a glycine-serine linker. In further embodiments, the linker polypeptide comprises, or alternatively consists essentially thereof, or further consists of the sequence (glycine-serine)n wherein n is an integer from 1 to 6.

**[0159]** Also provided are isolated nucleic acids that encode the CAR constructs. The nucleic acids can further comprise the necessary regulatory sequences, e.g., a promoter for expression in a host cell, e.g., a mammalian or human host cell such as a T cell. In one aspect the promoter is a CMV, MND, or an EF1alpha promoter. In a further aspect, the CAR polynucleotide futher comprises a marker peptide (e.g., GFP) that may be regulated from a second promoter element, e.g, CMV, MND, and EF1A promoters, located 5' to the encoding polynucleotide. In one aspect, the second promoter comprises an EF1 alpha promoter. As is apparent to the skilled artisan, the promoter(s) are selected for the host expression system and wil vary with the host and the expresson vector and intended use.

**[0160]** The isolated nucleic acid can be inserted into an expression vector, e.g., a lentiviral vector or retroviral vector ( between the 5' and 3' LTRs) or an adenovirus vector or any other vectors that can express a gene from. Figure 1A is an exemplary construct of this disclosure. As is apparent, when used clinically in a human patient, marker or purification tags will be omitted from the construct.

***Process for Preparing Antibodies***

**[0161]** Antibodies for use in this disclosure can be purchased or prepared using methods known in the art and briefly described herein. If a new antigen is discovered, it will be necessary to manufacture antibodies and antigen binding domains of the antibodies. Their manufacture and uses are well known and disclosed in, for example, Harlow, E. and Lane, D., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1999. The antibodies may be generated using standard methods known in the

art. Examples of antibodies include (but are not limited to) monoclonal, single chain, and functional fragments of antibodies.

**[0162]** Antibodies may be produced in a range of hosts, for example goats, rabbits, rats, mice, humans, and others. They may be immunized by injection with a target antigen or a fragment or oligopeptide thereof which has immunogenic properties, such as a C-terminal fragment a cancer or tumor relevant antigen or an isolated polypeptide, such as BCMA or NKG2D. Depending on the host species, various adjuvants may be added and used to increase an immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (Bacille Calmette-Guerin) and *Corynebacterium parvum* are particularly useful. This this disclosure also provides the isolated polypeptide and an adjuvant.

**[0163]** In certain aspects, the antibodies of the present disclosure are polyclonal, *i.e.*, a mixture of plural types of antibodies having different amino acid sequences. In one aspect, the polyclonal antibody comprises a mixture of plural types of antibodies having different CDRs. As such, a mixture of cells which produce different antibodies is cultured, and an antibody purified from the resulting culture can be used (see WO 2004/061104).

**[0164]** *Monoclonal Antibody Production*. Monoclonal antibodies to a cancer or tumor antigen may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. Such techniques include, but are not limited to, the hybridoma technique (*see,* e.g., Kohler & Milstein, *Nature* 256: 495-497 (1975)); the trioma technique; the human B-cell hybridoma technique (*see,* e.g., Kozbor et al., *Immunol. Today* 4: 72 (1983)) and the EBV hybridoma technique to produce human monoclonal antibodies (*see, e.g.*, Cole et al., in: MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96 (1985)). Human monoclonal antibodies can be utilized in the practice of the present technology and can be produced by using human hybridomas (*see,* e.g., Cote et al., *Proc. Natl. Acad. Sci.* 80: 2026-2030 (1983)) or by transforming human B-cells with Epstein Barr Virus *in vitro* (*see,* e.g., Cole et al., in: MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96 (1985)). For example, a population of nucleic acids that encode regions of antibodies can be isolated. PCR utilizing primers derived from sequences encoding conserved regions of

antibodies is used to amplify sequences encoding portions of antibodies from the population and then reconstruct DNAs encoding antibodies or fragments thereof, such as variable domains, from the amplified sequences. Such amplified sequences also can be fused to DNAs encoding other proteins—e.g., a bacteriophage coat, or a bacterial cell surface protein—for expression and display of the fusion polypeptides on phage or bacteria. Amplified sequences can then be expressed and further selected or isolated based, e.g., on the affinity of the expressed antibody or fragment thereof for an antigen or epitope present on the BCMA relevant antigen polypeptide. Alternatively, hybridomas expressing monoclonal antibodies can be prepared by immunizing a subject, e.g., with an isolated polypeptide comprising, or alternatively consisting essentially of, or yet further consisting of, the amino acid sequence of the relevant antigen or a fragment thereof, and then isolating hybridomas from the subject's spleen using routine methods. *See,* e.g., Milstein et al., (Galfre and Milstein, *Methods Enzymol* 73: 3-46 (1981)). Screening the hybridomas using standard methods will produce monoclonal antibodies of varying specificity (i.e., for different epitopes) and affinity. A selected monoclonal antibody with the desired properties, e.g., a relevant antigen binding, can be (i) used as expressed by the hybridoma, (ii) bound to a molecule such as polyethylene glycol (PEG) to alter its properties, or (iii) a cDNA encoding the monoclonal antibody can be isolated, sequenced and manipulated in various ways. In one aspect, the monoclonal antibody is produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, e.g., a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell. Hybridoma techniques include those known in the art and taught in Harlow et al., *Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 349 (1988); Hammerling et al., *Monoclonal Antibodies And T-Cell Hybridomas,* 563-681 (1981).

**[0165]** *Phage Display Technique.* As noted above, the antibodies of the present disclosure can be produced through the application of recombinant DNA and phage display technology. For example, BCMA antibodies, can be prepared using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of a phage particle which carries polynucleotide sequences encoding them. Phage with a desired binding property is selected from a repertoire or combinatorial antibody library (e.g., human or murine) by selecting directly with an antigen, typically an antigen bound or captured to a solid surface or bead. Phage used in these

methods are typically filamentous phage including fd and M13 with Fab, $F_v$ or disulfide stabilized $F_v$ antibody domains are recombinantly fused to either the phage gene III or gene VIII protein. In addition, methods can be adapted for the construction of Fab expression libraries (*see,* e.g., Huse et al., *Science* 246: 1275-1281, 1989) to allow rapid and effective identification of monoclonal Fab fragments with the desired specificity for a relevant antigen polypeptide, e.g., a polypeptide or derivatives, fragments, analogs or homologs thereof. Other examples of phage display methods that can be used to make the isolated antibodies of the present disclosure include those disclosed in Huston et al., *Proc. Natl. Acad. Sci. U.S.A.,* 85: 5879-5883 (1988); Chaudhary et al., *Proc. Natl. Acad. Sci. U.S.A.,* 87: 1066-1070 (1990); Brinkman et al., *J. Immunol. Methods* 182: 41-50 (1995); Ames et al., *J. Immunol. Methods* 184: 177-186 (1995); Kettleborough *et al.*, *Eur. J. Immunol.* 24: 952-958 (1994); Persic et al., *Gene* 187: 9-18 (1997); Burton et al., *Advances in Immunology* 57: 191-280 (1994); PCT/GB91/01134; WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; WO 96/06213; WO 92/01047 (Medical Research Council *et al.*); WO 97/08320 (Morphosys); WO 92/01047 (CAT/MRC); WO 91/17271 (Affymax); and U.S. Patent Nos. 5,698,426, 5,223,409, 5,403,484, 5,580,717, 5,427,908, 5,750,753, 5,821,047, 5,571,698, 5,427,908, 5,516,637, 5,780,225, 5,658,727 and 5,733,743.

**[0166]** Methods useful for displaying polypeptides on the surface of bacteriophage particles by attaching the polypeptides *via* disulfide bonds have been described by Lohning, U.S. Patent No. 6,753,136. As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host including mammalian cells, insect cells, plant cells, yeast, and bacteria. For example, techniques to recombinantly produce Fab, Fab′ and $F(ab')_2$ fragments can also be employed using methods known in the art such as those disclosed in WO 92/22324; Mullinax et al., *BioTechniques* 12: 864-869 (1992); Sawai et al., *AJRI* 34: 26-34 (1995); and Better et al., *Science* 240: 1041-1043 (1988).

**[0167]** Generally, hybrid antibodies or hybrid antibody fragments that are cloned into a display vector can be selected against the appropriate antigen in order to identify variants that maintained good binding activity, because the antibody or antibody fragment will be present on the surface of the phage or phagemid particle. *See,* e.g., Barbas III et al., *Phage*

*Display, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001). However, other vector formats could be used for this process, such as cloning the antibody fragment library into a lytic phage vector (modified T7 or Lambda Zap systems) for selection and/or screening.

**[0168]** *Alternate Methods of Antibody Production.* Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents (Orlandi et al., *PNAS* 86: 3833-3837 (1989); Winter, G. et al., *Nature*, 349: 293-299 (1991)).

**[0169]** Alternatively, techniques for the production of single chain antibodies may be used. Single chain antibodies (scFvs) comprise a heavy chain variable region and a light chain variable region connected with a linker peptide (typically around 5 to 25 amino acids in length). In the scFv, the variable regions of the heavy chain and the light chain may be derived from the same antibody or different antibodies. scFvs may be synthesized using recombinant techniques, for example by expression of a vector encoding the scFv in a host organism such as *E. coli.* DNA encoding scFv can be obtained by performing amplification using a partial DNA encoding the entire or a desired amino acid sequence of a DNA selected from a DNA encoding the heavy chain or the variable region of the heavy chain of the above-mentioned antibody and a DNA encoding the light chain or the variable region of the light chain thereof as a template, by PCR using a primer pair that defines both ends thereof, and further performing amplification combining a DNA encoding a polypeptide linker portion and a primer pair that defines both ends thereof, so as to ligate both ends of the linker to the heavy chain and the light chain, respectively. An expression vector containing the DNA encoding scFv and a host transformed by the expression vector can be obtained according to conventional methods known in the art.

**[0170]** Antigen binding fragments may also be generated, for example the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse et al., *Science*, 256: 1275-1281 (1989)).

**[0171]** *Antibody Modifications.* The antibodies of the present disclosure may be multimerized to increase the affinity for an antigen. The antibody to be multimerized may

be one type of antibody or a plurality of antibodies which recognize a plurality of epitopes of the same antigen. As a method of multimerization of the antibody, binding of the IgG CH3 domain to two $scF_v$ molecules, binding to streptavidin, introduction of a helix-turn-helix motif and the like can be exemplified.

**[0172]** The antibody compositions disclosed herein may be in the form of a conjugate formed between any of these antibodies and another agent (immunoconjugate). In one aspect, the antibodies disclosed herein are conjugated to radioactive material. In another aspect, the antibodies disclosed herein can be bound to various types of molecules such as polyethylene glycol (PEG).

**[0173]** *Antibody Screening.* Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between the relevant antigen, or any fragment or oligopeptide thereof and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies specific to two non-interfering relevant antigen epitopes may be used, but a competitive binding assay may also be employed (Maddox et al., *J. Exp. Med.*, 158: 1211-1216 (1983)).

**[0174]** *Antibody Purification.* The antibodies disclosed herein can be purified to homogeneity. The separation and purification of the antibodies can be performed by employing conventional protein separation and purification methods.

**[0175]** By way of example only, the antibody can be separated and purified by appropriately selecting and combining use of chromatography columns, filters, ultrafiltration, salt precipitation, dialysis, preparative polyacrylamide gel electrophoresis, isoelectric focusing electrophoresis, and the like. *Strategies for Protein Purification and Characterization: A Laboratory Course Manual*, Daniel R. Marshak et al. eds., Cold Spring Harbor Laboratory Press (1996); *Antibodies: A Laboratory Manual.* Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988).

**[0176]** Examples of chromatography include affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography, reverse

phase chromatography, and adsorption chromatography. In one aspect, chromatography can be performed by employing liquid chromatography such as HPLC or FPLC.

**[0177]** In one aspect, a Protein A column or a Protein G column may be used in affinity chromatography. Other exemplary columns include a Protein A column, Hyper D, POROS, Sepharose F. F. (Pharmacia) and the like.

***Bispecific Antibodies***

**[0178]** Also provided herein are bispecific antibody constructs that comprises any antidies of NKG2D or of a tumor antigen, e.g., antigen binding domain of an anti-NKG2D antibody and a tumor targeting antigen binding domain of an antibody. The antigen binding domains can be from any appropriate species, e.g., murine, human or a humanized sequence. In one aspect, the tumor targeting antibody comprises anti-CS1 or anti-BCMA. It also includes any molecues binds to these two proteins. For example, MICA, MICB, and ULBPs. In one aspect, the CAR can be CS1 CAR and the bispecific part can be replaced with anti-BCMA scFv. In one aspect, the tumor targeting antibody comprises anti-CS1 or anti-BCMA. An example of such is described in PCT/US2016/018955, filed February 22, 2016, incorporated herein by reference specifically incuding the polynucleotide and amino acid sequence of such.

**[0179]** In one aspect, at least one of the two antigen binding domains is specific for an antigen that is co-expressed with a predetermined first antigen. For example, in the case of MM, BCMA and CS1 are co-expressed on MM, and CS1 was selected to compliment the BCMA antigen binding domain of a CAR construct. Alternatively, BCMA can compliment an anti-CS1 CAR.

**[0180]** In further aspect, the antigen binding domains comprise, or consist essentially of, or yet further consist of a scFv fragment, that is optionally codon-optimized. In a further aspect, the bispecific antigen binding domains are the variable heavy and light chains that are joined by a peptide linker. In general, the antigen binding domains can be joined together by a peptide linker, e.g., a non-immunogenic protein linker derived from human muscle aldose[35].

**[0181]** Also provided are isolated nucleic acids that encode the bispecific antibodies. The nucleic acids can further comprise the necessary regulatory sequences, e.g., a promoter for expression in a host cell, e.g., a mammalian or human host cell such as a T cell and/or an

enhancer. In one aspect the promoter is a CMV or an EF1 alpha promoter. The isolated nucleic acid can further comprise polynucleotides encoding detectable markers such as GFP that may be located downstream from the BsAb encoding polynucleotide and regulated from a separate promoter element, e.g., an EF1alpha promoter. As is apparent to the skilled artisan, the promoter(s) are selected for the host expression system.

**[0182]** The isolated nucleic acid can be inserted into an expression vector, e.g., a lentiviral vector, between the 5' and 3' LTRs. Figure 1B is an exemplary lentiviral vector construct of this disclosure. As is apparent to the skilled artisan, the constructs may not comprise a marker peptide or purification marker.

***BsAb-CAR Constructs***

**[0183]** In further aspect, provided herein are isolated nucleic acids that encode, in one construct, a CAR construct and a bispecific antibody as disclosed above ("BsAb-CAR construct"). The antigen binding domains can be from any appropriate species, e.g., murine, human or a humanized sequence. In one aspect, the isolated nucleic acid encodes an antigen binding fragment that targets BCMA and a bispecific antibody, e.g., one scFv from an anti-CS1 antibody and one scFV from an anti-NKG2D antibody, joined together by a nucleic acid encoding a non-immunogenic protein linker such as from human muscle aldose. In one aspect, the nucleic acid encoding the CAR construct is located 5' to the nucleic acid encoding the BsAb. In one aspect, a T2A element is located between the 5' located CAR polynucleotide and the 3' located BsAb. The nucleic acids can further comprise the necessary regulatory sequences, e.g., a promoter for expression in a host cell, e.g., a mammalian or human host cell such as a T cell. In one aspect the promoter is an EF1a or a CMV promoter located 5' to the polynucleotide encoding the CAR. In a further aspect, the isolated nucleic acid can further comprise polynucleotides encoding detectable markers such as GFP that may be downstream from the BsAb polynucleotide and under the control of a separate promoter element, e.g., an EF1alpha promoter. As is apparent to the skilled artisan, the promoter(s) are selected for the host expression system.

**[0184]** The isolated nucleic acid can be inserted into an expression vector, e.g., a lentiviral vector, between the 5' and 3' LTRs. Figure 3A is an exemplary lentiviral vector construct

of this disclosure. As is apparent to the skilled artisan, the constructs may not comprise a marker peptide or purification marker.

***Host Cells and Processes for Preparing CARs***

**[0185]** Aspects of the present disclosure relate to an isolated cell comprising a CAR, a BsAb, and a BsAb CAR, and methods of producing such cells. The cell is a prokaryotic or a eukaryotic cell. In one aspect, the cell is a T-cell, a B cell, a NK cell, a dendritic cell, a myeloid cell, a monocyte, a macrophage, any subsets thereof, or any other immune cell. The eukaryotic cell can be from any preferred species, e.g., an animal cell, a mammalian cell such as a human, a feline or a canine cell. The cells may be derived from patients, donors, or cell lines, such as those available off-the-shelf. The cells can be autologous or allogeneic to the subject being treated.

**[0186]** In specific embodiments, the isolated cell comprises, or alternatively consists essentially of, or yet further consists of an exogenous CAR or a BsAb CAR comprising, or alternatively consisting essentially of, or yet further consisting of, an antigen binding domain of a cancer or tumor antibody, a CD8 α hinge domain, a CD8 α transmembrane domain, a CD28 costimulatory signaling region and/or a 4-1BB costimulatory signaling region, and a CD3 zeta signaling domain. In a separate embodiment, the isolated cel further comprises a BsAb as discloed herein. In a yet further aspec, the cell comprises the BsAb as disclosed herein. In certain embodiments, the isolated cell is a T-cell, e.g., an animal T-cell, a mammalian T-cell, a feline T-cell, a canine T-cell or a human T-cell. In certain embodiments, the isolated cell is an NK-cell, e.g., an animal NK-cell, a mammalian NK-cell, a feline NK-cell, a canine NK-cell or a human NK-cell. In certain embodiments, the isolated cell is a B-cell, e.g., an animal B-cell, a mammalian B-cell, a feline B-cell, a canine B-cell or a human B-cell. It is appreciated that the same or similar embodiments for each species apply with respect to dendritic cells, myeloid cells, monocytes, macrophages, any subsets of these or the T-cells, NK-cells, and B-cells described, and/or any other immune cells. In one aspect, the cell is a T cell that has been modified to remove CD52 expression using gene editing technology, e.g., CRISPR or TALEN.

**[0187]** In certain embodiments, methods of producing the BsAb, CAR, and/or BsAb CAR expressing cells are disclosed the method comprising, or alternatively consisting essentially of or yet further consisting of transducing a population of isolated cells with a nucleic acid sequence encoding the BsAb, the CAR, the BsAb and CAR, and/or BsAb CAR. In a further

aspect, a subpopulation of cells that have been successfully transduced with the nucleic acid sequence is selected. In some embodiments, the isolated cells are T-cells, an animal T-cell, a mammalian T-cell, a feline T-cell, a canine T-cell or a human T-cell, thereby producing the BsAb, the CAR, the BsAb and CAR, and/or BsAb CAR T-cells. In certain embodiments, the isolated cell is an NK-cell, e.g., an animal NK-cell, a mammalian NK-cell, a feline NK-cell, a canine NK-cell or a human NK-cell, thereby producing the BsAb, the CAR, the BsAb and CAR, and/or BsAb CAR NK-cells. In some embodiments, the isolated cells are B-cells, an animal B-cell, a mammalian B-cell, a feline B-cell, a canine B-cell or a human B-cell, thereby producing the BsAb, the CAR, the BsAb and CAR, and/or BsAb CAR B-cells. It is appreciated that the same or similar embodiments for each species apply with respect to dendritic cells, myeloid cells, monocytes, macrophages, any subsets of these or the T-cells, NK-cells, and B-cells described, and/or any other immune cells. In one aspect, the cell is a T cell that has been modified to remove CD52 expression using gene editing technology, e.g., CRISPR or TALEN. In one aspect, the cells are autologous or allogenic to the subject being treated.

**[0188]** *Sources of Isolated Cells.* Prior to expansion and genetic modification of the cells disclosed herein, cells may be obtained from a subject – for instance, in embodiments involving autologous therapy – or a commercially available cell line or culture, or a stem cell such as an induced pluripotent stem cell (iPSC).

**[0189]** Cells can be obtained from a number of sources in a subject, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors.

**[0190]** Methods of isolating relevant cells are well known in the art and can be readily adapted to the present application; an exemplary method is described in the examples below. Isolation methods for use in relation to this disclosure include, but are not limited to Life Technologies Dynabeads® System; STEMcell Technologies EasySep™, RoboSep™, RosetteSep™, SepMate™; Miltenyi Biotec MACS™ cell separation kits, and other commercially available cell separation and isolation kits. Particular subpopulations of immune cells may be isolated through the use of beads or other binding agents available in such kits specific to unique cell surface markers. For example, MACS™ CD4+ and CD8+ MicroBeads may be used to isolate CD4+ and CD8+ T-cells. Alernate non-limiting

examples of cells that may be isolated accoriding to known techniques include bulked T-cells, NK T-cells, and gamma delta T-cells.

**[0191]** Alternatively, cells may be obtained through commercially available cell cultures, including but not limited to, for T-cells, lines BCL2 (AAA) Jurkat (ATCC® CRL-2902™), BCL2 (S70A) Jurkat (ATCC® CRL-2900™), BCL2 (S87A) Jurkat (ATCC® CRL-2901™), BCL2 Jurkat (ATCC® CRL-2899™), Neo Jurkat (ATCC® CRL-2898™); for B cells, lines AHH-1 (ATCC® CRL-8146™), BC-1 (ATCC® CRL-2230™), BC-2 (ATCC® CRL-2231™), BC-3 (ATCC® CRL-2277™), CA46 (ATCC® CRL-1648™), DG-75 [D.G.-75] (ATCC® CRL-2625™), DS-1 (ATCC® CRL-11102™), EB-3 [EB3] (ATCC® CCL-85™), Z-138 (ATCC #CRL-3001), DB (ATCC CRL-2289), Toledo (ATCC CRL-2631), Pfiffer (ATCC CRL-2632), SR (ATCC CRL-2262), JM-1 (ATCC CRL-10421), NFS-5 C-1 (ATCC CRL-1693); NFS-70 C10 (ATCC CRL-1694), NFS-25 C-3 (ATCC CRL-1695), and SUP-B15 (ATCC CRL-1929); and, for NK cells, lines NK-92 (ATCC® CRL-2407™), NK-92MI (ATCC® CRL-2408™). Further examples include but are not limited to mature T-cell lines, e.g., Deglis, EBT-8, HPB-MLp-W, HUT 78, HUT 102, Karpas 384, Ki 225, My-La, Se-Ax, SKW-3, SMZ-1 and T34; immature T- cell lines, *e.g.*, ALL-SIL, Be13, CCRF-CEM, CML-T1, DND-41, DU.528, EU-9, HD-Mar, HPB-ALL, H-SB2, HT-1, JK-T1, Jurkat, Karpas 45, KE-37, KOPT-K1, K-T1, L-KAW, Loucy, MAT, MOLT-1, MOLT 3, MOLT-4, MOLT 13, MOLT-16, MT-1, MT-ALL, P12/Ichikawa, Peer, PER0117, PER-255, PF-382, PFI-285, RPMI-8402, ST-4, SUP-T1 to T14, TALL-1, TALL-101, TALL-103/2, TALL-104, TALL-105, TALL-106, TALL-107, TALL-197, TK-6, TLBR-1, -2, -3, and -4, CCRF-HSB-2 (CCL-120.1), J.RT3-T3.5 (ATCC TIB-153), J45.01 (ATCC CRL-1990), J.CaM1.6 (ATCC CRL-2063), RS4;11 (ATCC CRL-1873), CCRF-CEM (ATCC CRM-CCL-119); cutaneous T-cell lymphoma lines, e.g., HuT78 (ATCC CRM-TIB-161), MJ[G11] (ATCC CRL-8294), HuT102 (ATCC TIB-162); B-cell lines deived from anaplastic and large cell lymphomas, e.g., DEL, DL-40, FE-PD, JB6, Karpas 299, Ki-JK, Mac-2A Ply1, SR-786, SU-DHL-1, -2, -4,-5,-6,-7,-8,-9,-10, and -16, DOHH-2, NU-DHL-1, U-937, Granda 519, USC-DHL-1, RL; Hodgkin's lymphomas, e.g., DEV, HD-70, HDLM-2, HD-MyZ, HKB-1, KM-H2, L 428, L 540, L1236, SBH-1, SUP-HD1, and SU/RH-HD-l; and NK lines such as HANK1, KHYG-1, NKL, NK-YS, NOI-90, and YT. Null leukemia cell lines, including but not limited to REH, NALL-1, KM-3, L92-221, are a another commercially available source of immune cells, as are cell lines derived from other leukemias and lymphomas, such as K562 erythroleukemia, THP-1 monocytic leukemia,

U937 lymphoma, HEL erythroleukemia, HL60 leukemia, HMC-1 leukemia, KG-1 leukemia, U266 myeloma. Non-limiting exemplary sources for such commercially available cell lines include the American Type Culture Collection, or ATCC, (atcc.org/) and the German Collection of Microorganisms and Cell Cultures (dsmz.de/).

**[0192]** In some embodiments, T-cells expressing the disclosed CARs may be further modified to reduce or eliminate expression of endogenous TCRs. Reduction or elimination of endogenous TCRs can reduce off-target effects and increase the effectiveness of the T cells. T cells stably lacking expression of a functional TCR may be produced using a variety of approaches. T cells internalize, sort, and degrade the entire T cell receptor as a complex, with a half-life of about 10 hours in resting T cells and 3 hours in stimulated T cells (von Essen, M. et al. 2004. J. Immunol. 173:384-393). Proper functioning of the TCR complex requires the proper stoichiometric ratio of the proteins that compose the TCR complex. TCR function also requires two functioning TCR zeta proteins with ITAM motifs. The activation of the TCR upon engagement of its MHC-peptide ligand requires the engagement of several TCRs on the same T cell, which all must signal properly. Thus, if a TCR complex is destabilized with proteins that do not associate properly or cannot signal optimally, the T cell will not become activated sufficiently to begin a cellular response.

**[0193]** Accordingly, in some embodiments, TCR expression may eliminated using RNA interference (e.g., shRNA, siRNA, miRNA, etc.), CRISPR, or other methods that target the nucleic acids encoding specific TCRs (e.g., TCR-α and TCR-β) and/or CD3 chains in primary T cells. By blocking expression of one or more of these proteins, the T cell will no longer produce one or more of the key components of the TCR complex, thereby destabilizing the TCR complex and preventing cell surface expression of a functional TCR. Even though some TCR complexes can be recycled to the cell surface when RNA interference is used, the RNA (e.g., shRNA, siRNA, miRNA, etc.) will prevent new production of TCR proteins resulting in degradation and removal of the entire TCR complex, resulting in the production of a T cell having a stable deficiency in functional TCR expression.

**[0194]** Expression of inhibitory RNAs (e.g., shRNA, siRNA, miRNA, etc.) in primary T cells can be achieved using any conventional expression system, e.g., a lentiviral expression system. Although lentiviruses are useful for targeting resting primary T cells, not all T cells will express the shRNAs. Some of these T cells may not express sufficient amounts of the

RNAs to allow enough inhibition of TCR expression to alter the functional activity of the T cell. Thus, T cells that retain moderate to high TCR expression after viral transduction can be removed, e.g., by cell sorting or separation techniques, so that the remaining T cells are deficient in cell surface TCR or CD3, enabling the expansion of an isolated population of T cells deficient in expression of functional TCR or CD3.

**[0195]** Expression of CRISPR in primary T cells can be achieved using conventional CRISPR/Cas sytems and guide RNAs specific to the target TCRs. Suitable expression systems, e.g. lentiviral or adenoviral expression systems are known in the art. Similar to the delivery of inhibitor RNAs, the CRISPR system can be use to specifically target resting primary T cells or other suitable immune cells for CAR cell therapy. Further, to the extent that CRISPR editing is unsuccessful, cells can be selected for success according to the methods disclosed above. For example, as noted above, T cells that retain moderate to high TCR expression after viral transduction can be removed, e.g., by cell sorting or separation techniques, so that the remaining T cells are deficient in cell surface TCR or CD3, enabling the expansion of an isolated population of T cells deficient in expression of functional TCR or CD3. It is further appreciated that a CRISPR editing construct may be useful in both knocking out the endogenous TCR and knocking in the CAR constructs disclosed herein. Accordingly, it is appreciated that a CRISPR system can be designed for to accomplish one or both of these purposes.

**[0196]** *Vectors*. CAR cells may be prepared using vectors. Aspects of the present disclosure relate to an isolated nucleic acid sequence encoding (i) a CAR or (ii) a polynucleotide encoding an immunoregulatory molecule and vectors comprising, or alternatively consisting essentially of, or yet further consisting of, an either one or both of these nucleic acids and/or complements and/or equivalents of each thereof.

**[0197]** In some embodiments, the isolated nucleic acid sequence encodes for a CAR comprising, or alternatively consisting essentially of, or yet further consisting of an antigen binding domain of a cancer or tumor targeting antibody, a CD8 α hinge domain, a CD8 α transmembrane domain, a CD28 costimulatory signaling region and/or a 4-1BB costimulatory signaling region, and a CD3 zeta signaling domain. In specific embodiments, the isolated nucleic acid sequence comprises, or alternatively consisting essentially thereof, or yet further consisting of, sequences encoding (a) an antigen binding domain of a cancer or tumor targeting antibody followed by (b) a CD8 α hinge domain, (c) a CD8 α

transmembrane domain followed by (d) a CD28 costimulatory signaling region and/or a 4-1BB costimulatory signaling region followed by (e) a CD3 zeta signaling domain.

**[0198]** In some embodiments, the isolated nucleic acid sequence encodes for a CAR and comprises, or alternatively consists essentially of, or yet further consists of, a Kozak consensus sequence upstream of the sequence encoding the antigen binding domain of the cancer or tumor targeting antibody.

**[0199]** In one aspect, the antigen binding domain targets BCMA.

**[0200]** In some embodiments, the isolated nucleic acid comprises, or alternatively consists essentially of, or yet further consists of a polynucleotide encoding a bispecific antibody. In certain embodiments, the bispecific antibody, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to NKG2D, and optionally, SLAMF7 (also known as CS1 or CD319), that are optionally codon optimized, or an equivalent of each thereof. In certain embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319) (that are optionally codon optimized) or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the heavy chain and/or light chain variable region of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319) (that are optionally codon optimized) and/or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises a single chain variable fragment (scFV) derived from an antibody to NKG2D and, optionally, a single chain variable fragment (scFV) derived from SALMF7 (also known as CS1 of CD319) (that are optionally codon optimized) and/or an equivalent each thereof. In some embodiments, the isolated nucleic acid comprises, or alternatively consists essentially of, or yet further consists of a polynucleotide sequence encoding the bispeicifc antibody operatively linked to a promoter that may be generated according to the method disclosed above.

**[0201]** In some embodiments, the isolated nucleic acid comprises a detectable label and/or a polynucleotide conferring antibiotic resistance. In one aspect, the label or polynucleotide are useful to select cells successfully transduced with the isolated nucleic acids.

**[0202]** In some embodiments, the isolated nucleic acid sequence is comprised within a vector. In certain embodiments, the vector is a plasmid. In other embodiments, the vector is a viral vector. Non-limiting examples of such include without limitation a retroviral vector, a lentiviral vector, an adenoviral vector, and an adeno-associated viral vector. In specific embodiments, the vector is a lentiviral vector.

**[0203]** The preparation of exemplary vectors and the generation of CAR expressing cells using said vectors is discussed in detail in the examples below. In summary, the expression of natural or synthetic nucleic acids encoding CARs or immunoregulatory molecules is typically achieved by operably linking a nucleic acid encoding the CAR polypeptide or portions thereof to a promoter, and incorporating the construct into an expression vector. A similar method may be used to construct the isolated nucleic acid sequence comprising a polynucleotide encoding an immunoregulatory molecule. The vectors can be suitable for replication and integration eukaryotes. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

**[0204]** In one aspect, the term "vector" intends a recombinant vector that retains the ability to infect and transduce non-dividing and/or slowly-dividing cells and integrate into the target cell's genome. In several aspects, the vector is derived from or based on a wild-type virus. In further aspects, the vector is derived from or based on a wild-type lentivirus. Examples of such include without limitation, human immunodeficiency virus (HIV), equine infectious anemia virus (EIAV), simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV). Alternatively, it is contemplated that other retrovirus can be used as a basis for a vector backbone such murine leukemia virus (MLV). It will be evident that a viral vector according to the disclosure need not be confined to the components of a particular virus. The viral vector may comprise components derived from two or more different viruses, and may also comprise synthetic components. Vector components can be manipulated to obtain desired characteristics, such as target cell specificity.

**[0205]** The recombinant vectors of this disclosure are derived from primates and non-primates. Examples of primate lentiviruses include the human immunodeficiency virus (HIV), the causative agent of human acquired immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the

prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV). Prior art recombinant lentiviral vectors are known in the art, e.g., see U.S. Patent Nos. 6,924,123; 7,056,699; 7,419,829 and 7,442,551, incorporated herein by reference.

**[0206]** U.S. Patent No. 6,924,123 discloses that certain retroviral sequence facilitate integration into the target cell genome. This patent teaches that each retroviral genome comprises genes called gag, pol and env which code for virion proteins and enzymes. These genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. They also serve as enhancer-promoter sequences. In other words, the LTRs can control the expression of the viral genes. Encapsidation of the retroviral RNAs occurs by virtue of a psi sequence located at the 5' end of the viral genome. The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA, and U5 is derived from the sequence unique to the 5'end of the RNA. The sizes of the three elements can vary considerably among different retroviruses. For the viral genome. and the site of poly (A) addition (termination) is at the boundary between R and U5 in the right hand side LTR. U3 contains most of the transcriptional control elements of the provirus, which include the promoter and multiple enhancer sequences responsive to cellular and in some cases, viral transcriptional activator proteins.

**[0207]** With regard to the structural genes gag, pol and env themselves, gag encodes the internal structural protein of the virus. Gag protein is proteolytically processed into the mature proteins MA (matrix), CA (capsid) and NC (nucleocapsid). The pol gene encodes the reverse transcriptase (RT), which contains DNA polymerase, associated RNase H and integrase (IN), which mediate replication of the genome.

**[0208]** For the production of viral vector particles, the vector RNA genome is expressed from a DNA construct encoding it, in a host cell. The components of the particles not encoded by the vector genome are provided in trans by additional nucleic acid sequences (the "packaging system", which usually includes either or both of the gag/pol and env genes) expressed in the host cell. The set of sequences required for the production of the viral vector particles may be introduced into the host cell by transient transfection, or they

may be integrated into the host cell genome, or they may be provided in a mixture of ways. The techniques involved are known to those skilled in the art.

**[0209]** Retroviral vectors for use in this disclosure include, but are not limited to Invitrogen's pLenti series versions 4, 6, and 6.2 "ViraPower" system. Manufactured by Lentigen Corp.; pHIV-7-GFP, lab generated and used by the City of Hope Research Institute; "Lenti-X" lentiviral vector, pLVX, manufactured by Clontech; pLKO.1-puro, manufactured by Sigma-Aldrich; pLemiR, manufactured by Open Biosystems; and pLV, lab generated and used by Charité Medical School, Institute of Virology (CBF), Berlin, Germany.

**[0210]** Further methods of introducing exogenous nucleic acids into the art are known and include but are not limited to gene delivery using one or more of RNA electroporation, nanotechnology, sleeping beauty vectors, retroviruses, and/or adenoviruses.

**[0211]** Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise expose a cell to the inhibitor of the present disclosure, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the disclosure.

**[0212]** *Packaging vector and cell lines.* The isolated nucleic acids can be packaged into a retroviral packaging system by using a packaging vector and cell lines. The packaging vector includes, but is not limited to retroviral vector, lentiviral vector, adenoviral vector, and adeno-associated viral vector. The packaging vector contains elements and sequences that facilitate the delivery of genetic materials into cells. For example, the retroviral constructs are packaging vectors comprising at least one retroviral helper DNA sequence derived from a replication-incompetent retroviral genome encoding in trans all virion proteins required to package a replication incompetent retroviral vector, and for producing virion proteins capable of packaging the replication-incompetent retroviral vector at high titer, without the production of replication-competent helper virus. The retroviral DNA sequence lacks the region encoding the native enhancer and/or promoter of the viral 5′ LTR of the virus, and lacks both the psi function sequence responsible for packaging helper

genome and the 3′ LTR, but encodes a foreign polyadenylation site, for example the SV40 polyadenylation site, and a foreign enhancer and/or promoter which directs efficient transcription in a cell type where virus production is desired. The retrovirus is a leukemia virus such as a Moloney Murine Leukemia Virus (MMLV), the Human Immunodeficiency Virus (HIV), or the Gibbon Ape Leukemia virus (GALV). The foreign enhancer and promoter may be the human cytomegalovirus (HCMV) immediate early (IE) enhancer and promoter, the enhancer and promoter (U3 region) of the Moloney Murine Sarcoma Virus (MMSV), the U3 region of Rous Sarcoma Virus (RSV), the U3 region of Spleen Focus Forming Virus (SFFV), or the HCMV IE enhancer joined to the native Moloney Murine Leukemia Virus (MMLV) promoter. The retroviral packaging vector may consist of two retroviral helper DNA sequences encoded byplasmid based expression vectors, for example where a first helper sequence contains a cDNA encoding the gag and pol proteins of ecotropic MMLV or GALV and a second helper sequence contains a cDNA encoding the env protein. The Env gene, which determines the host range, may be derived from the genes encoding xenotropic, amphotropic, ecotropic, polytropic (mink focus forming) or 10A1 murine leukemia virus env proteins, or the Gibbon Ape Leukemia Virus (GALV env protein, the Human Immunodeficiency Virus env (gp160) protein, the Vesicular Stomatitus Virus (VSV) G protein, the Human T cell leukemia (HTLV) type I and II env gene products, chimeric envelope gene derived from combinations of one or more of the aforementioned env genes or chimeric envelope genes encoding the cytoplasmic and transmembrane of the aforementioned env gene products and a monoclonal antibody directed against a specific surface molecule on a desired target cell.

**[0213]** In the packaging process, the packaging vectors and retroviral vectors are transiently cotransfected into a first population of mammalian cells that are capable of producing virus, such as human embryonic kidney cells, for example 293 cells (ATCC No. CRL1573, ATCC, Rockville, Md.) to produce high titer recombinant retrovirus-containing supernatants. In another method of the disclosure this transiently transfected first population of cells is then cocultivated with mammalian target cells, for example human lymphocytes, to transduce the target cells with the foreign gene at high efficiencies. In yet another method of the invention the supernatants from the above described transiently transfected first population of cells are incubated with mammalian target cells, for example human lymphocytes or hematopoietic stem cells, to transduce the target cells with the foreign gene at high efficiencies.

**[0214]** In another aspect, the packaging vectors are stably expressed in a first population of mammalian cells that are capable of producing virus, such as human embryonic kidney cells, for example 293 cells. Retroviral or lentiviral vectors are introduced into cells by either cotransfection with a selectable marker or infection with pseudotyped virus. In both cases, the vectors integrate. Alternatively, vectors can be introduced in an episomally maintained plasmid. High titer recombinant retrovirus-containing supernatants are produced.

**[0215]** *Activation and Expansion of CAR Cells.* Whether prior to or after genetic modification of the cells to express a desirable CAR, the cells can be activated and expanded using generally known methods such as those described in U.S. Patent Nos. 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681 ; 7, 144,575; 7,067,318; 7, 172,869; 7,232,566; 7, 175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041 and references such as Lapateva et al. (2014) Crit Rev Oncog 19(1-2):121-32; Tam et al. (2003) Cytotherapy 5(3):259-72; Garcia-Marquez et al. (2014) Cytotherapy 16(11):1537-44. Stimulation with the tumor relevant antigen *ex vivo* can activate and expand the selected CAR expressing cell subpopulation. Alternatively, the cells may be activated *in vivo* by interaction with a tumor relevant antigen.

**[0216]** In the case of certain immune cells, additional cell populations, soluble ligands and/or cytokines, or stimulating agents may be required to activate and expand cells. The relevant reagents are well known in the art and are selected according to known immunological principles. For instance, soluble CD-40 ligand may be helpful in activating and expanding certain B-cell populations; similarly, irradiated feeder cells may be used in the procedure for activation and expansion of NK cells.

**[0217]** Methods of activating relevant cells are well known in the art and can be readily adapted to the present application; an exemplary method is described in the examples below. Isolation methods for use in relation to this disclosure include, but are not limited to Life Technologies Dynabeads® System activation and expansion kits; BD Biosciences Phosflow™ activation kits, Miltenyi Biotec MACS™ activation/expansion kits, and other commercially available cell kits specific to activation moieties of the relevant cell. Particular subpopulations of immune cells may be activated or expanded through the use of beads or other agents available in such kits. For example, α-CD3/α-CD28 Dynabeads® may be used to activate and expand a population of isolated T-cells.

***Methods of Use***

**[0218]** *Therapeutic Application.* Method aspects of the present disclosure relate to methods for inhibiting the growth of a tumor or cancer cells, (e.g., MM cells) in vitro or in vivo and/or for treating a cancer patient in need thereof. In some embodiments, the tumor is a solid tumor. In some embodiments, the cancer is a cancer affecting blood and/or bone marrow, e.g., MM. In some embodiments, the cancer or tumor cell expresses or overexpresses a cancer or tumor antigen, e.g., BCMA and/or CS1. When practiced *in vitro*, the methods provide in vitro assays for precision medicine application and useful assays for testing new combination and therapies.

**[0219]** In certain embodiments, these methods comprise, or alternatively consist essentially of, or yet further consist of, administering to the subject or patient an effective amount of the isolated cell comprising the CAR. In further embodiments, this isolated cell comprises or expresses a CAR and/or a bispecific antibody. In some embodiments, the antigen binding domain of the CAR comprises, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to any one of B-cell maturation antigen (BCMA) and/or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof. In some embodiments, the antigen binding domain of the CAR comprises, or alternatively consists essentially thereof, or further consists of the heavy chain and/or light chain variable region of an antibody to any one of B-cell maturation antigen (BCMA) and/or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof. In still further embodiments, the isolated cell is a T-cell or an NK cell. In some embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of an NKG2D ligand and, optionally, a SALMF7 (also known as CS1 of CD319) ligand. In certain embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the heavy chain and/or light chain variable region of an antibody to NKG2D and, optionally, SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises a single chain variable fragment (scFV) derived from an antibody to NKG2D and, optionally, a single chain variable fragment (scFV) derived from SALMF7

(also known as CS1 of CD319), and/or an equivalent each thereof. In some embodiments, the isolated cell is autologous to the subject or patient being treated. In a further aspect, the tumor expresses a cancer or tumor antigen and the subject has been selected for the therapy by a diagnostic, such as use of a antibody that recognizes and binds the tumor or cancer relevant antigens targeted by the CARs. The subject is an animal, a mammal, a canine, a feline, a bovine, an equine, a murine or a human patient.

**[0220]** The CAR cells as disclosed herein may be administered either alone or in combination with the bispecific antibody disclosed herein, diluents, known anti-cancer therapeutics, and/or with other components such as cytokines or other cell populations that are immunoregulatory. They can be administered as a first line therapy, a second line therapy, a third line therapy, or further therapy. Non-limiting examples of additional therapies include cytoreductive therapy, such as radiation therapy, cryotherapy, or chemotherapy, or biologics. Further non-limiting examples include other relevant cell types, such as unmodified immune cells, modified immune cells comprising vectors expressing one or more immunoregulatory molecules, or CAR cells specific to a different antigen than those disclosed herein. As with the CAR cells of the present disclosure, in some embodiments, these cells may be autologous or allogenic. Appropriate treatment regimens will be determined by the treating physician or veterinarian.

**[0221]** Pharmaceutical compositions of the present disclosure may be administered in a manner appropriate to the disease to be treated or prevented. The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials. In one aspect they are administered directly by direct injection or systemically such as intravenous injection.

**[0222]** Aspects of the disclosure provide an exemplary method for determining if a patient is likely to respond to, or is not likely to respond to, CAR therapy. The method comprises, or alternatively consists essentially thereof, or further consists of determining the presence or absence of necrosis in a tumor sample isolated from the patient and quantitating the amount of cancer or tumor cells expressin the cancer or tumor antigen. In certain embodiments, the method further comprises, or alternatively consists essentially of, or yet further consists of administering an effective amount of the CAR therapy to the patient that is determined likely to respond to the CAR therapy. The CAR therapy can be autologous or

allogenic to the patient and the patient can be subject that suffers from a solid tumor, animal or human.

**[0223]** Techniques of histological staining for necrosis are well known in the art. For example, hematoxylin and eosin stains, also referred to as "H&E staining," are a common technique for identifying the presence of necrosis in tissues, especially in tumorigenic or cancerous growth. Cytoplasmic H&E staining demonstrates increased eosinophilia, attributable in part to the loss of cytoplasmic RNA and in part to denatured cytoplasmic proteins. In necrotic tissue stains, the cytoplasm often appears "moth eaten" due to enzyme digestion of cytoplasmic organelles. Myelin figures, calcification, and evidence of phagocytosis into other cells are also hallmarks of necrotic tissues that can be detected by histological staining. Necrotic tissues also have specific hallmarks in nuclear staining often demonstrating karyolysis, pyknosis, and karyorrhexis as a result of cell death. Using microscopy and either manual or automated quantitation of such necrotic hallmarks, relevance of CAR therapy may be determined. Alternate means of detecting tumorigenic or cancerous growth or necrotic tissues in general, including but not limited to biomarker-based or imaging-based diagnostics, are also equally relevant to determining whether a patient will respond to certain types of CAR therapy, and may be used accordingly.

***Carriers***

**[0224]** Additional aspects of the disclosure relate to compositions comprising, or alternatively consisting essentially of, or yet further consisting of, a carrier and one or more of the products – e.g., a CAR, an isolated cell comprising a CAR, an isolated nucleic acid, a vector, an isolated cell containing the CAR and the bispecific antibody disclosed herein and/or nucleic acids encoding such – described in the embodiments disclosed herein. In further aspects, the composition may additionally comprise an immunoregulatory molecule and/or an isolated nucleic acid comprising a polynucleotide encoding a bispecific antibody. In certain embodiments, the bispecific antibody, or alternatively consists essentially thereof, or further consists of the relevant CDR regions of an antibody to BCMA and/or NKG2D, optionally, SLAMF7 (also known as CS1 or CD319), or an equivalent of each thereof. In some embodiments, the bispecific antibody comprises, or alternatively consists essentially thereof, or further consists of the heavy chain and/or light chain variable region of an antibody to NKG2D, optionally, SLAMF7 (also known as CS1 or CD319) (that are optionally codon optimized) and/or an equivalent of each thereof. In some embodiments,

the bispecific antibody comprises a single chain variable fragment (scFv) derived from an antibody to NKG2D, optionally, a single chain variable fragment (scFv) derived from SALMF7 (also known as CS1 of CD319) (that are optionally codon optimized) and/or an equivalent each thereof.

**[0225]** Briefly, pharmaceutical compositions of the present disclosure including but not limited to any one of the claimed compositions as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the present disclosure may be formulated for oral, intravenous, topical, enteral, and/or parenteral administration. In certain embodiments, the compositions of the present disclosure are formulated for intravenous administration.

**[0226]** Administration of the cells or compositions can be effected in one dose, continuously or intermittently throughout the course of treatment and an effective amount to achieve the desired therapeutic benefit is provided. Methods of determining the most effective means and dosage of administration are known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents are known in the art. In a further aspect, the cells and composition of the disclosure can be administered in combination with other treatments.

**[0227]** The cells and populations of cell are administered to the host using methods known in the art and described, for example, in PCT/US2011/064191. This administration of the cells or compositions of the disclosure can be done to generate an animal model of the desired disease, disorder, or condition for experimental and screening assays.

***Combination Therapies***

**[0228]** The compositions as described herein can be administered as first line, second line, third line, fourth line, or other therapy and can be combined with cytoreductive interventions. The can be administered sequentially or concurrently as determined by the

treating physician. In one aspect, they can be combined with therapies that may upregulate the expression of a tumor or other antigen to which the CAR and/or BsAb binds. In one aspect, some clinical drugs can increase targeted antigens. For example, CS1 surface expression can be increased by Lenalidomide, an immune modulator drug for mutiple myloma that is FDA-approved, see Wang et al. (2018) Clin. Cancer Res. Jan 1;24(1):106-119. Another example is the FDA-approved drug midostaurin that increases FLT3 expression when the CAR-BsAb targets a FLT3 antigen.

***Kits***

**[0229]** As set forth herein, the present disclosure provides methods for producing and administering CAR and/or BsAb CAR cells. In one particular aspect, the present disclosure provides kits for performing these methods as well as instructions for carrying out the methods of the present disclosure such as collecting cells and/or tissues, and/or performing the screen/transduction/etc., and/or analyzing the results.

**[0230]** In one aspect the kit comprises, or alternatively consists essentially of, or yet further consists of, any one of the isolated nucleic acids disclosed herein and/or a vector comprising said nucleic acid and/or isolated allogenic cells, preferably T cells or NK cells, and/or instructions on the procuring of autologous cells from a patient. Such a kit may also comprise, or alternatively consist essentially of, or yet further comprise media and other reagents appropriate for the transduction and/or selection and/or activation and/or expansion of CAR and/or BsAb CAR expressing cells, such as those disclosed herein.

**[0231]** In one aspect the kit comprises, or alternatively consists essentially of, or yet further consists of, an isolated CAR and/or BsAb CAR expressing cell or population thereof. In some embodiments, the cells of this kit may require activation and/or expansion prior to administration to a subject in need thereof. In further embodiments, the kit may further comprise, or consist essentially thereof, media and reagents, such as those covered in the disclosure above, to activate and/or expand the isolated CAR and/or BsAb CAR expressing cell. In some embodiments, the cell is to be used for CAR therapy. In further embodiments, the kit comprises instructions on the administration of the isolated cell to a patient in need of CAR therapy.

**[0232]** The kits of this disclosure can also comprise, e.g., a buffering agent, a preservative or a protein-stabilizing agent. The kits can further comprise components necessary for

detecting the detectable-label, e.g., an enzyme or a substrate. The kits can also contain a control sample or a series of control samples, which can be assayed and compared to the test sample. Each component of a kit can be enclosed within an individual container and all of the various containers can be within a single package, along with instructions for interpreting the results of the assays performed using the kit. The kits of the present disclosure may contain a written product on or in the kit container. The written product describes how to use the reagents contained in the kit.

**[0233]** As amenable, these suggested kit components may be packaged in a manner customary for use by those of skill in the art. For example, these suggested kit components may be provided in solution or as a liquid dispersion or the like.

**[0234]** The following examples are illustrative of procedures which can be used in various instances in carrying the disclosure into effect.

**EXAMPLE 1 – BsAb CAR T-cells Generation and Efficacy**

**[0235]** Chimeric antigen receptor (CAR) T cells and bispecific antibodies (BsAb) are FDA-approved therapies and show impressive curative potential for cancer. However, in the majority of cases, neither have yet been shown to be curative. This could be due in part to the duration of the therapies, i.e., CAR T cells may not survive sufficiently long *in vivo,* and BsAb have a very short half-life with a costly and time-consuming manufacturing process, thus limiting their efficacy and broad application. Here, Applicants successfully created a platform to combine CAR T cell therapy with BsAb therapy, both of which have potential for long-lasting effects. Applicants tested this platform in the setting of multiple myeloma (MM), an incurable cancer with high rates of relapse following currently FDA-approved therapies. Applicants validated the concept utilizing two MM target antigens, CS1 and BCMA, and created a novel and effective single lentiviral construct to generate BsAb-CAR T cells expressing a BCMA CAR as well as secreting a soluble anti-NKG2D-anti-CS1 BsAb, with the former attacking BCMA(+) MM tumor cells and the latter engaging all NKG2D(+) cytolytic cells including CD8(+) T cells, γδ T cells, natural killer (NK) T cells, and NK cells, for the purpose of promoting their cytotoxicity activity against CS1(+) MM. This all-in-one, multifaceted immune modality provides two "living drugs" simultaneously, i.e., CAR T cells and BsAb, capturing both innate and adaptive immune effector cells directed at different target antigens on the same malignant population. Applicants found that, compared with BCMA CAR T cells or BsAb transduced-T cells, BsAb-CAR T cells

secreted more IFN-γ and showed higher capacity for degranulation, while displaying enhanced cytotoxicity *in vitro* through targeting MM tumor cells, including MM cell lines and primary MM tumor cells. Ectopically forced expression of BCMA and CS1 in target cells lacking endogenous expression of these two antigens enhanced target cell lysis. Importantly, the anti-NKG2D-anti-CS1 BsAb secreted from the BCMA CAR T cells acts in an autocrine manner to trigger the BCMA CAR T cell proliferation *in vitro* and their enhanced proliferatio and survival *in in vivo*, respectively, through activation of NKG2D signaling. These multipronged effects resulted in strong anti-tumor activity *in vivo*. Collectively, provided herein is evidence for generating next-generation cancer immunotherapy, with a capacity to combine CAR T cell therapy and anti-NKG2D bispecific antibody therapy into a single platform for increased duration and enhanced efficacy as well as the ability to capture specific anti-tumor activity of both innate and adaptive cytolytic effector cells.

**[0236] Cell culture:** Cell lines, MM.1S, H929, RPMI-8226 (human multiple myeloma cell lines), and K562 (human erythroleukemic cell line) were purchased from the ATCC (Manassas, VA, USA). These cells were cultured with RPMI 1640 media (Sigma, St. Louis, USA) containing 10% fetal bovine serum (FBS) (Invitrogen, CA, USA) and 1% Antibiotic-Antimycotic (Invitrogen). The 293T cell line, which was purchased from ATCC and used for lentiviral production, was cultured in DMEM (Sigma) plus the same supplements as in RPMI 1640. Human peripheral blood mononuclear cells (PBMCs) from healthy donors and MM patients were isolated by Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Pittsburgh, PA) density gradient centrifugation, following the manufacturer's instructions. Human $CD56^+$ NK cells, $CD3^+CD56^+$ NKT cells, and $CD3^+$γöTCR$^+$ T cells were isolated using human NK, NKT and γöT cell isolation kits (MACS, Miltenyi Biotech, Auburn, CA, USA), respectively, according to the manufacturer's instructions. Primary MM patient samples were provided by the Leukemia Tissue Bank Shared Resource of the OSU Comprehensive Cancer Center and James Cancer Hospital. All work with human subjects was performed according to a protocol approved by The Ohio State University Institutional Review Board.

**[0237] Mice:** Six- to 8-week-old NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were used for all *in vivo* studies. All animal work was performed according to a protocol approved by The Ohio State University Animal Care and Use Committee. The progression of MM disease was

closely monitored, and survival data were recorded. The mice were sacrificed upon observation of hind limb paralysis, lethargy, and obvious weight loss.

**[0238] Generation of BCMA-CAR, anti-NKG2D-anti-CS1 BsAb, and BsAb-BCMA CAR- lentiviral constructs:** For BCMA CAR, the BCMA coding domain sequences for variable regions of heavy (VH) and light (VL) chains were derived from a hybridoma and recombined using a linker. The VH-linker-VL fragment was incorporated in frame with the CD28–CD3zeta portion. The anti-BCMA-scFv-CD28-CD3zeta fragment was subcloned into the lentiviral vector pCDH to create a second-generation pCDH-BCMA CAR construct. To make the anti-NKG2D-anti-CS1 BsAb lentiviral construct, two codon-optimized single chain variable fragments (scFv) from an anti-CS1 monoclonal antibody[29] and an anti-NKG2D antibody joined together by a non-immunogenic protein linker derived from human muscle aldose[35] were cloned into the pCDH lentiviral vector. For "all in one" BsAb-BCMA CAR, the anti-BCMA-scFv-CD28-CD3ζ -T2A cassette was incorporated into the pCDH anti-CS1-NKG2D BsAb-EF1a-GFP to build a complete pCDH-BCMA CAR-T2A-BsAb-EF1a-GFP lentiviral construct.

**[0239] Letiviral production and transduction of T cells:** Lentiviral transfection and infection were performed as described in a previously reported protocol [36,37].

**[0240] Generation of K562 cells stably expressing the CS1 and BCMA genes:** The full length pCDH-CMV-CS1-EF1α-GFP construct containing human CS1 coding sequences was previously reported[30]. To produce lentivirus, 293T cells were co-transfected with the pCDH-CS1 plasmid or a pCDH empty vector plasmid plus the packaging plasmids pCMV-VSVG and pCMV-δr9 using lipofectamine® 2000 (Invitrogen). Then the lentiviral supernatants were harvested and used to infect K562 cells using a previously published protocol [36,38]. GFP-positive cells were then sorted using an FACS Aria II cell sorter (BD Biosciences, San Jose, CA, USA). BCMA-K562 are K562 cells transduced with a vector carrying the full-length BCMA cDNA. Lentivirus production, infection and sorting were performed using the methods described above. $CS1^+BCMA^+$K562 cells were generated by transducing a pCDH-CMV-BCMA-EF1α-GFP lentiviral construct to CS1-K562 cells described above. The double-transduced cells were stained with an APC-anti-BCMA mAb and then sorted for $GFP^+BCMA^+$ population. Before being used for experiments, these $GFP^+BCMA^+$ double positive cells were passed several times in culture to ensure the loss of anti-BCMA mAb-bound cells.

**[0241] Flow Cytometry Analysis:** Detection of CAR expression on the cell surface was performed as previously reported [30]. Antibodies used in this study include: FITC and biotin-labeled goat anti-mouse (Fab)2 polyclonal antibody or normal polyclonal goat immunoglobulin G (IgG) antibody (Jackson ImmunoResearch), allophycocyanin (APC)-conjugated streptavidin (Jackson ImmunoResearch), PerCP/Cy5.5-conjugated streptavidin (Biolegend), PE, PerCP/Cy5.5 andBV421 anti-human CD3 (hCD3, clone UCHT1 and SK7, BD Biosciences), APC and PE anti-hCD56 (clone TULY56 and CMSSB, eBioscience), FITC and PC5.5 anti-TCR pan γ/δ (clone IMMU510, Beckman Coulter, Inc. CA, USA), PC5 anti-TCR pan α/β (clone IP26A, Beckman), FITC anti-TCR Vγ9 (clone IMMU 360, Beckman) and Pacific Blue anti-TCR Vδ2 (clone IMMU 389, Beckman), unconjugated and APC-anti hNKG2D (clone 1D11BD Biosciences), PE-Cy7 anti-hCD8 (clone SK1, BD Biosciences), APC-Cy7 anti-hCD4 (clone SK3, BD Biosciences), BV421 anti-human CD1d (clone CD1d42, BD OptiBuild), V450 anti-hCD11c (clone B-ly6, BD Horizon), APC-H7 anti-hCD19 (clone HIB19, BD Pharmingen), APC-H7 anti-hCD20 (clone 2H7, BD Biosciences), FITC anti-hCD45 (clone J.33, Beckman), biotin and FITC anti-hCD14 (clone 63D3 and M5E2, Biolegend), biotin and PE anti-hCD33 (clone P67.6, Biolegend, and WM53, BD Pharmingen), biotin anti-hCD66b (clone G10F5, Biolegend), unconjugated and PE-anti hCS1 (clone 162, eBioscience), APC-anti hBCMA (clone 19F2, Biolegend), PE-anti Ki67 (clone SolA15, BD Biosciences), PE-hCD69 (clone FN50, BD Biosciences). Cells were washed once with PBS containing 4% bovine serum albumin, stained with antibodies for 20 min at room temperature, and analyzed with a LSRII flow cytometer (BD Biosciences, San Jose, CA, USA).

**[0242] Immunoblotting:** To detect intracellular expression and secretion of the bi-specific antibody, BsAb-transduced T cells or BsAb-CAR T cells and cell-free supernatants from the culture of these cells were collected for immunoblotting using 6x-his-tagged mAb (clone 4A12E4, Invitrogen). Immunoblotting was performed according to a standard immunoblotting protocol that Applicants previously reported [30,37]. For the detection of CAR expression, CAR-transduced T cells were lysed and proteins were extracted for immunoblotting, probing with mouse anti-human CD3ζ mAb (BD Pharmingen), as previously reported [30,37].

**[0243] Cytotoxicity Assay:** cells were labeled with $^{51}Cr$ and co-cultured with transduced T cells at various effector: target ratios (E:T) in the wells of 96-well V-bottom plates at 37

°C for 4 h, followed by harvesting supernatants to measure the release of $^{51}Cr$ from target cells using TopCount counter (Canberra Packard). When studying the capacity of BsAb to engage PBMC, $CD3^+$T cells, $CD8^+$cytotoxic T cells, γδ T cells, NKT cells, and NK cells were isolated from leukopacks ordered from the American Red Cross. T cells were primed with Dynabeads™ Human T-Activator CD3/CD28 ($4 \times 10^4$/ μL, Beads:T=1:1) with IL-2 (500 U/mL) and IL-15 (500 U/mL) for 5-14 days prior to the standard 4-hour $^{51}Cr$ release assay described above. Human NK cells were activated by IL-2 (500 U/mL) for prior to cytotoxicity assay. Isolated human $CD3^+CD56^+$ NKT cells were activated by α-GalCer (α-Galactosylceramide, KRN7000, Enzo Biochem Inc. NY, USA. 100ng/mL) with IL-2 (100U/mL) [39] for 7-10 days. For $CD3^+\gamma\delta TCR^+$ T cells activation, HMBPP ((E)-1-Hydroxy-2-methyl-2-butenyl 4-pyrophosphate, Sigma. 10nM) with IL-2 (100U/mL) [40,41] were used and cultured for 14 days. The protocol for isolation of these cells from leukopacks was approved by The Ohio State University Institutional Review Board.

**[0244] ELISA:** The presence of human IFN-γ in culture supernatants was assayed by enzyme-linked immunosorbent assay (ELISA) using a kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's protocol. The levels of human IL-2 and TNF-α in culture supernatants were also assayed by ELISA kits (Thermo Fisher Scientific, MA, USA). For these ELISA analyses, $2.5 \times 10^5$ cells of either a myeloma cell line or primary MM cells from patients were incubated with $2.5 \times 10^5$ engineered or control T cells in 96-well V bottom plates for 24 h. Data were read at 450 nm using a Synergy HT microplate reader (Biotek, Winooski, VT, USA).

**[0245] In vivo treatment of MM-bearing mice and bioluminescence imaging:** MM.1S myeloma cells expressing a firefly luciferase gene, MM.1S-GL3, have been previously described[30]. To build a xenograft orthotopic MM model, NSG mice (male) were injected with $8 \times 10^6$ MM.1S-GL3 cells in 200 μL of saline through tail-vein i.v. on day 0. On days 10, 17 and 24, mice were administered (1) vehicle control (saline) or $10 \times 10^6$ effector cells, including (2) empty-vector transduced T cells, (3) BsAb-transduced T cells, (4) BCMA–CAR-transduced T cells, (5) T cells sequentially transduced with BsAb and BCMA-CAR, or (6) BsAb-CAR-transduced T cells, by tail-vein i.v. injection, each in 200 μL saline. Ten days, 24 days, 31 days after inoculation with MM.1S-GL3 cells, D-luciferin (150 mg/kg body weight; Gold Biotechnology) was intraperitoneally injected to all mice. Imaging was performed using the In Vivo Imaging System (IVIS) with Living Image software

(PerkinElmer). On day 80, we challenged the surviving mice by tail-vein i.v injection of $4 \times 10^6$ MM1.S cells per mouse in 200 µl sline, Survival data were collected and closed on day 140. To investigate the effect of BsAb, the above experiment was repeated in the presence of PBMC with depletion of human myeloid cells. For this purpose, NSG mice were i.v. injected with $8 \times 10^6$ MM.1S-GL3 cells in 200 mL of saline via tail vein on day 0. On day 10, the mice were administered with $3 \times 10^6$ various transduced-T cells followed by $3 \times 10^6$ $CD33^-CD14^-CD66b^-$ human PBMC, all i.v. On day 17 and 24, the mice received $3 \times 10^6$ engineered T cells i.v. generated from the same donor. On day 10, day 19, day 28, and day 37, the mice were infused with D-luciferin and imaged as described above.

**[0246] Immune-synapse detected by immunofluorescence microscopy:** Primarily to label the secreted anti-NKG2D-anti-CS1 BsAb, supernatant from BsAb CAR T cells were collected and stained by 6x-His Tag mAb (clone 4E3D10H2/E3, Invitrogen) at a dilution of 1:500 for 1h in $37^0$C incubation and then labeled with Alexa Fluor®350 (blue, Thermo Fisher Scientific, MA, USA). MM.1S cells were harvested and incubated 45 min under growth conditions with CellTracker™ Deep Red Dye (20µM, Thermo Fisher Scientific). To see the immune-synapse, BsAb-CAR T cells (GFP, green) or empty vector-transduced control T cells (GFP, green) were co-cultured with MM.1S cells (red) and His Tag labeled supernatant for 1h or 24h. Live-cell fluorescence imaging were observed by Zeiss Microscope Systems (Zeiss Axio Observer Z1, Carl Zeiss Inc., NY, USA). For video shooting, BsAb-CAR T cells (GFP, green) or empty vector-transduced T cells (GFP, green) were co-cultured with MM.1S cells (red) for 1 h, and microscopy was used for observing immune-synapse during a period of 2 h.

**[0247] Results:** *Generation of primary T cells expressing BCMA-specific CAR and/or the the anti-NKG2D-anti-CS1 bispecific antibody*: Applicants generated a specific BCMA-CAR construct with a lentiviral vector backbone, which sequentially consists of a signal peptide (SP), a heavy chain variable region (VH), a glycine-serine (GS) linker, a light chain variable region (VL), a Myc tag, a hinge, CD28, and CD3ζ (Figure 1A). Next, applicants designed the anti-NKG2D-anti-CS1 bispecific antibody (referred to as "BsAb") construct with a lentiviral vector backbone. It consisted of two codon-optimized single chain variable fragments (scFv) from an anti-NKG2D antibody and an anti-CS1 monoclonal antibody, joined together by a non-immunogenic protein linker derived from human muscle aldose. Each scFv contains a corresponding heavy chain (VH) and light chain (VL) connected by a

glycine-serine (GS) linker (Figure 1B). The same donor T cells isolated from a healthy donor and activated by anti-human CD3/CD28 antibody beads were transduced with the empty vector (EV), the BsAb construct, BCMA-CAR construct, or first transduced with the the BsAb construct followed sequentially by transduction with the BCMA-CAR construct (heretoafter referred to as the BsAb-BCMA seq. trans. T construct). The expression of BCMA CAR on the cell surface was demonstrated by staining transduced T cells with anti-Fab, which detected expression of the scFv on more than 80% of FACS-enriched T cells transduced with either the BCMA CAR construct or the BsAb-BCMA seq. trans. T cell construct, whereas the expression remained almost undetectable on unmodified T cells, on EV-transduced T cells and on BsAb T cells (Figure 1C). To determine whether BsAb T cells and the BsAb-BCMA seq. trans. T cells were successfully transduced, cell-free supernatants from a 4-day culture were harvested and cell pellets from a 4-day culture were lysed. Both the supernatants and cell lysates were then subjected to immunoblotting using a 6x-his tagged Ab. Results showed that BsAb-T cells and BsAb-BCMA seq. trans. T cells produced both cellular and secreted BsAb while the controls from unmodified T cell supernatants and lysates, did not produce BsAb (Figure 1D).

**[0248]** *BsAb-BCMA seq. trans. T cells are more effective killers of MM than were T cells transduced with each vector alone in vitro*: Since the BsAb contained an anti-NKG2D receptor portion and an anti-CS1 portion, applicants attempted to trigger NKG2D activation on the $NKG2D^+$ cytolytic immune cells and tested whether it simultaneously engaged MM cells via the MM-associated antigen, CS1. Applicants first evaluated the surface expression of CS1 and BCMA in three commonly used MM cell lines MM.1S, H929, RPMI-8226 and a human erythroleukemic cell line, K562, by flow cytometric analysis. The results showed varied levels of BCMA and CS1 expression on the four MM cell lines. The MM1.S MM cell line has high levels of expression of both BCMA and CS1; the H929 MM cell line has high levels of BCMA and intermediate (int) levels of CS1 expression; and the RPMI-8226 MM cell line has intermediate levels of BCMA expression, while its CS1 expression is very low. As a negative control, the K562 erythroleukemia cell line did not express CS1 nor BCMA on the cell surface (Figure 2A). To determine whether the aforementioned BsAb-BCMA seq. trans. T cells (with sequentially transduced BCMA CAR and anti-NKG2D-anti-CS1 BsAb) could lead to more efficient tumor cell lysis of the MM cell lines, a standard 4-hour $^{51}Cr$-release assay was performed, using the K562 erythroleukemia cell line as negative target control. Prior to generating a single construct-engineered BsAb-CAR T

cell, Applicants compared MM tumor cell killing in five groups of T cells: (1) unmodified T cells, (2) empty vector (EV)-transduced T cells (EV T), (3) anti-NKG2D-anti-CS1-BsAb-transduced T cells (hereafter referred to as BsAb T), (4) BCMA-CAR-transduced T cells (hereafter referred to as BCMA-CAR T), and (5) anti-NKG2D-anti-CS1 BsAb and BCMA-CAR sequentially transduced T cells (referred to as BsAb-BCMA seq. trans. T). For the target MM cell lines $BCMA^{high}CS1^{high}$ MM.1S and $BCMA^{high}CS1^{int}$ H929, the BsAb-BCMA seq. trans. T cells produced significantly better killing than the BsAb T cells or BCMA-CAR T cells. For the target MM cell line $BCMA^{int}CS1^{low}$ RPMI-8226, the BsAb-BCMA seq. trans. T cells performed better than BsAb T cells but not better than BCMA-CAR T cells; Importantly, either single or combination antigen targeting had no activity against the negative control K562 erythroleukemic target cell line (Figure 2B). Of note, BCMA-CAR T cells were more effective at lysing MM target cells when compared to the effects of BsAb T cells, EV T cells, and unmodified T cells. Statistical analysis indicated that synergistic effects were observed for cytotoxicity assays conducted with the BsAb-BCMA seq. trans. T cell population of effector cells when the target MM cell lines expressed medium to high levels of CS1 and BCMA (i.e., MM1.S and H929), while this effect was not observed when target cells expressed low or medium levels of the two antigens (i.e., RPMI-8226) (Figure 2B).

**[0249]** To further determine the activation of the above transduced-T cells upon recognition of MM cells that endogenously express CS1 and BCMA, the Applicants measured IFN-γ, IL-2 and TNF-α secretion via ELISA in supernatants from unmodified T cells, EV T cells, BCMA-CAR T cells, BsAb T cells, and BsAb-BCMA seq. trans. T cells. IFN-γ secretion from BsAb-BCMA seq. trans. T cells was significantly higher than BsAb T cells or BCMA CAR T cells alone when co-cultured with $BCMA^{high}CS1^{high}$ MM.1S or $BCMA^{high}CS1^{int}$ H929 MM cell lines. In the co-cultured conditions with the $BCMA^{int}CS1^{low}$ RPMI-8226 MM cell line, IFN-γ secretion from BsAb-BCMA seq. trans. T cells was significantly higher than BsAb T cells but not significantly higher compared to BCMA T cells. In the co-culture condition with the $CS1^{-}BCMA^{-}$ K562 erythroleukemia cell line or no tumor target cells, BsAb-BCMA seq. trans. T cells were not superior to single antigen targeting with either BsAb T cells or BCMA-CAR T cells (Figure 2C). IL-2 production in BsAb T cells, BCMA-CAR T cells or BsAb-BCMA seq. trans. T cells was dramatically higher than unmodified T cells or EV T cells when co-cultured with MM.1S,

H929 or RPMI-8226 MM cell lines, (Figure 2D). Interestingly, even in co-cultures with the negative control K562 erythroleukemia cell line or no target cells, BsAb T cells and BsAb-BCMA seq. trans. T cells secreted a high level of IL-2 that was significantly higher than IL-2 secretion seen in BCMA CAR T cells (Figure 2D), suggesting that at least in this instance, the effect appears to result from the presence of the secreted BsAb itself, rather than from the MM target cells. TNF-α secretion was consistent with IFN-γ secretion (Figure 2E). Overall, these results indicated that, compared with BsAb T cells or BCMA-CAR T cells, BsAb-BCMA seq. trans. T cells can more specifically recognize MM target cells, and become more activated after the recognition of these MM cells. Moreover, compared to other conditions, Applicants also noticed that the BsAb secreted from BsAb T cells or BsAb-BCMA seq. trans. T cells appeared to trigger T cell activation regardless of the presence or absence of MM cells, as evidenced by their highly abundant IL-2 production compared to T cells not expressing the BsAb construct (Figure 2D). This suggested that the NKG2D receptor expressed on cytotoxic CD8(+) T cells was being activated by the presence of the secreted BsAb.

**[0250]** *Generation of single construct-engineered BsAb-CAR T cells to target both BCMA and CS1 in MM*: Applicants noted that T cells co-expressing BCMA-CAR and anti-NKG2D-anti-CS1 BsAb delivered by two separate constructs (i.e., BsAb-BCMA seq. trans. T cells) were superior at killing MM cells when compared to T cells expressing either BCMA-CAR or BsAb. A single construct expressing both a BsAb and a CAR (referred to hereafter as BsAb-CAR) would be more practical in (1) producing effective expression of both constructs in a single T cell; (2) decreasing manufacturing costs; and (3) saving time. Applicants therefore generated a single BsAb-CAR construct containing both parts in a lentiviral vector backbone connected by T2A (Figure 3A). To generate primary T cells expressing BsAb-CAR, Applicants utilized the same method described above and then determined whether the BsAb-CAR-transduced T cells were successfully transduced. The surface expression of the CAR was confirmed by flow cytometric analysis (Figure 21C). The BsAb fusion protein was successfully detected using a 6x-his-tagged Ab on day 4 in both cell lysates and in the serum-free-medium (Figure 3B). Additionally, to dynamically measure BsAb secretion, applicants re-seeded the BsAb-CAR T cells in serum-free medium on day 5, and then collected cell-free supernatants at 12h, 24h, 48h, 72h and 96h. The results showed that the BsAb secretion was potent, as expression started before 12h and continued to increase in a time dependent manner (Figure 21A-B). Applicants then

demonstrated that unfractionated BsAb-CAR T cells showed significantly superior killing of the $BCMA^{high}CS1^{high}$ MM cell line MM.1S compared to killing by EV T cells and unmodified T cells (Figure 3C). Similar results were obtained against the $BCMA^{high}CS1^{int}$ H929 and $BCMA^{int}CS1^{low}$ RPMI-8226 MM target cell lines, while there was no killing against the negative control K562 erythroleukemia cell line (Figure 21D). As approximately 80% of $CD8^+$ T cells have high surface density expression of NKG2D (vs. 30% of unfractionated T cells; Figure 11A), Applicants transduced to highly enriched $CD8^+$ T cells with the BsAb-CAR construct. Perhaps predictably, Applicants found these cells to have higher cytotoxicity than $CD8^+$ BsAb T cells and $CD8^+$ BCMA-CAR T cells as well as higher than two negative controls, unmodified T cells and EV T cells, against the $BCMA^{high}CS1^{high}$ MM.1S MM cell line (Figure 3D).

**[0251]** The BsAb secreted by CAR T cells requires two antigens to be functional: CS1 expressed on tumor cells, and NKG2D expressed on immune cells. Applicants first assessed the percentages of TCR pan $\alpha/\beta$ $CD3^+T$, TCR pan $\gamma/\delta$ $CD3^+T$, $CD3^+CD56^+NKT$, and $CD3^-CD56^+NK$ cells among PBMC, which represent approximately 50%, 1%, 8%, and 15% of PBMC, respectively (Figure 11A). Furthermore, Applicants confirmed NKG2D is expressed on approximately 30% of T cells, 80% of $CD8^+T$ cells, 70% of $\gamma\delta$ T cells, 60% of NKT cells, and 90% NK cells. Of note, nearly 90% of $V\gamma9V\delta2$ T cells, which are a subset of $\gamma\delta$ T cells, expressed NKG2D (Figures 11B and 11C). To determine whether anti-NKG2D-anti-CS1 BsAb triggers $NKG2D^+$ immune cells, Applicants undertook 4-hour chromium-51 release assays as described above at the ratio of 10 Effector (transduced or unmodified T cells) to 1 target cells (MM.1S), but added different quantities of human PBMC, i.e., 1-, 10-, 100-, or 200-fold of tumor cells. Applicants hypothesized that if the secreted BsAb from either the BsAb T cells and/or the BsAb-CAR T cells were recruiting non-transduced NKG2D+ cytolytic effector cells to the CS1+ MM cell line target, the killing of the target would go up with greater dilution from the addition of non-transduced PBMC. In support of their hypothesis, as non-transduced PBMC were increased, only cultures containing transduced T cells that secreted the BsAb (i.e., BsAb T cells and BsAb-CAR T cells) showed increasing cytotoxicity against the $BCMA^{high}CS1^{high}$ MM.1S MM cell line (Figure 3E). Predictably, the effect was more modest against the $BCMA^{high}CS1^{int}$ H929 cell line with lower expression of CS1 than the MM.1S MM cell line and was absent against the $BCMA^{int}CS1^{low}$ RPMI-8226 MM target cell line, Figure 12A, 12B).

**[0252]** Applicants next enriched each subset of PBMC, i.e., NK cells, NKT cells, $CD8^+$ T cells, and γ9Vδ2 T cells, to nearly 98% purity (Figure 13), and priming them with IL-2, CalCer plus IL-2, CD3/CD28 Dynabeads plus IL-2, and HMBPP plus IL-2, respectively, and transduced each with one of the control or experimental vectors, followed by a 4h (Figure 3F, left) or 16h (Figure 3F, right) $^{51}$Cr-release cytotoxicity assay against the $BCMA^{high}CS1^{high}$ MM.1S MM cell line at an E:T ratio of 5:1 for each. At four or more hours of incubation, the results showed that BsAb CAR T cells have significantly higher cytotoxicity than identical cells transduced with the other constructs, regardless of the T cell subset or the method of activation (Figures 3F and 14). Importantly, among the BsAb CAR T cell populations examined, compared to control cells, the activated $CD4^+$ BsAb CAR T cells had the least increase in cytotoxic activity (Figure 3F, right and Figure 14), likely due at least in part to the relatively low surface density expression of NKG2D on this population.

**[0253]** To determine whether BsAb secreted by BsAb-CAR T cells can induce synapse formation between BsAb-CAR T cells and MM.1S MM cells, a confocal microscopy analysis was conducted after one hour of co-incubation. When a control of co-culture of EV T cells and MM.1S MM cells was observed, no synapses were seen (Figure 3G). In contrast, synapses were observed during the co-culture of BsAb-CAR T cells and MM.1S MM cells (Figures 3H).

**[0254]** Further, when the 24 hours co-cultures of BsAb CAR-T cells (green) with the target MM.1S MM cells (red) were observed, Applicants noticed that only the BsAb CAR-T population (green) was present (Figure 15A-F); yet in the co-culture of EV T cells and MM.1S MM cells, both the target MM.1S MM cells and the effector EV T cells (green) were still present (Figure 15G-M).

**[0255]** *Functionally enhanced recognition and activation of BsAb-CAR T cells are CS1 and BCMA-dependent*: To prove that enhanced cytotoxic effect of BsAb-CAR T cells depended on targeting tumor antigens, Applicants next explored whether forced overexpression of CS1 and BCMA in the BsAb-CAR T cell-resistant K562 cell line could lower its threshold for lysis against this effector population. For this purpose, Applicants sequentially transduced to K562 cells with lentiviruses encoding human CS1 and BCMA (or empty vector PCDH as control) to generate the K562 cell line ectopically expressing CS1 and BCMA (Figure 16). After confirming the success of generating the target cell line

(Figure 4A), Applicants performed $^{51}Cr$ release assays and the results indicated that overexpression of CS1 and BCMA in the K562 erythroleukemia cell line resulted in a significant increase in the cytotoxic activity of BsAb-CAR T cells against this K562 cell line as compared to the cytotoxicity using the EV T cells (Figure 4B, purple lines). Applicants next performed ELISA assays, which showed an increase of IFN-γ and IL-2 secretion in co-cultures of BsAb-CAR T cells and K562-CS1-BCMA cells, compared to co-cultures of EV T cells with K562-CS1-BCMA cells (Figure 4C, 4D). However, there was no difference in cytotoxicity, IFN-γ and IL-2 production between BsAb-CAR T cells and EV T cells when they were incubated with K562-PCDH (Figure 4B-D). Collectively, these data suggested that the increased recognition, killing, and cytokine secretion of target cells by BsAb-CAR T cells occurred in a CS1 and BCMA-dependent manner.

**[0256]** *Secreted anti-NKG2D-anti-CS1 BsAb enhances CAR T cell proliferation in vitro and both survival and proliferation in vivo through NKG2D signaling*: In some patients, CAR T cells do not survive very long, due to limited expansion and survival capacity, and this can limit the efficacy of these cells [42]. Unexpectedly, Applicants found that the culture medium of BsAb-transduced T cells and BsAb-CAR T cells was more acidotic compared to other conditions without BsAb at similar time points, suggesting a higher rate of cell metabolism (Figure 5A, top). This observation is consistent with the data described above that these BsAb-transduced cells can secrete more cytokine even without target cells or with $BCMA^{-}CS1^{-}$ target cells (Figure 2D). Applicants speculated that expression and secretion of the BsAb can induce T cell proliferation, survival, and/or its activation. Cell enumeration confirmed that, compared to other culture conditions, those containing T cells transduced with the BsAb or BsAb-CAR construct indeed contained a significantly higher quantity of cells (Figure 5A, bar graph), which resulted from T cell proliferation as documented by the violet cell tracker and shown as V450 dilution displayed in histograms in the lower panel (Figure 5A, histogram).

**[0257]** To confirm these unexpected results, Applicants added the supernatants from BsAb-CAR T cell cultures shown in Figure 5A to cell culture conditions that were without BsAb, i.e., BCMA CAR T cells, EV T cells, and unmodified T cells. Indeed, adding supernatants from BsAb-CAR T cell cultures resulted in a significantly greater degree of cell metabolism after 5 days incubation, as evidenced by the yellow color of the medium as occurs in cell turnover, compared with cultures of BCMA CAR T cells, EV T cells, and

unmodified T cells that were not supplemented with supernatants from BsAb-CAR T cell medium (not shown). Cell enumeration confirmed that, compared to other culture conditions, those supplement with medium from BsAb-CAR T cell cultures indeed contained a significantly higher quantity of cells (Figure 5B, top), which resulted from T cell proliferation as documented by the violet cell tracker and shown as V450 dilution displayed in histograms in the lower panel (Figure 5B, bottom). Ki67 staining indicated that, in the cultures containing either BsAb T cells or BsAb-CAR T cells, the vast majority of $NKG2D^+$ cells were proliferating, as well as nearly half of $NKG2D^-$ cells (Figure 5C, and Figure 17A), indicating that BsAb-activated $NKG2D^+$ cells can in turn activate $NKG2D^-$ cells, albeit to a lesser degree. Moreover, NKG2D blocking antibody mitigated the proliferative effects of the secreted BsAb (Figure 5C blue frame, Figure 17B). An immunoblot analysis was performed to determine the phosphorylation (p) of AKT protein, confirming that secreted BsAb can trigger $NKG2D^+$ cell proliferation and activation under BsAb T and BsAb-CAR T cells culture conditions because these conditions have a higher level of p-AKT (Figure 5D).

**[0258]** The data presented thus far supported the notion that activation of the NKG2D+ immune cells by the BsAb was occurring through the NKG2D pathway. The Applicants next asked if activation was occurring via CS1, which is expressed not only on MM cells, but also on NK, NKT, $CD8^+$ T, and B cells or their sbsets. (Veillette and Guo Crit Rev Oncol Hematol. 2013 Oct;88(1):168-77. doi: 10.1016/j.critrevonc.2013.04.003. Epub 2013 Jun 2; Gogishvili et al. Blood. 2017 Dec 28;130(26):2838-2847. doi: 10.1182/blood-2017-04-778423. Epub 2017 Oct 31.) To determine whether secreted BsAb was also stimulating $NKG2D^+$ immune cells via their expression of CS1 and via the $CS1^+$ signaling pathway, Applicants next blocked this pathway using a anti-CS1 blocking antibody. Applicants found CS1 blockade did not affect the activation status of immune cells (Figure 17C). Moreover, Applicants performed NKG2D and CS1 double blocking, and results demonstrated that there was no difference compared with NKG2D single blockade (data not shown). Collectively, these data suggest that activation of immune cells secreting BsAb only occurs through the NKG2D signaling pathway.

**[0259]** Based on the above findings, Applicants had anticipated that in co-cultures of the T cells with the target population, MM.1S MM cells, immune activation would not be strictly dependent on NKG2D, nor restricted to the $NKG2D^+$ subset of T cells. For example,

when BsAb T and BsAb-CAR T cells were co-cultured with MM.1S MM cells, both $NKG2D^+$ (red in Figure 5E; 83% to 94%) and $NKG2D^-$ (green in Figure 5E; 79% to 87%) fractions of $CD3^+$ T cells showed extensive proliferation as measured by Ki67 staining. Likewise, for $CD3^+$ T cells only expressing the BCMA CAR (denoted in Figure 5E as $Fab^+$) both $NKG2D^+$ (Red; 90.4%) and $NKG2D^-$ (Green; 85.8%) fractions of $CD3^+$ T cells showed extensive proliferation as measured by Ki67 staining, albeit slightly less than was seen with both $NKG2D^+$ and $NKG2D^-$ BsAb-CAR T cells.

**[0260]** To investigate the ability of the transduced T cells to survive *in vitro*, Applicants cultured various transduced T cells in the presence or absence of IL-2. Under the IL-2 condition, all cells showed high Ki67 expression and low Annexin V and/or Sytox Blue expression (Figure 6a). Interestingly, in IL-2 deficient condition (Figure 6b), only the BsAb T cells and BsAb-CAR T cells (which secrete the BsAb and activate the T cells via NKG2D) showed better proliferation ability as about 80% Ki67 expression, which is consistent with data presented in Figure 2D, and with the reduced cell apoptosis and death as illustrated by the low expression of Annexin V and/or Sytox Blue staining (Figure 6b). These results are compared with the other three groups (unmodified T cells, EV T cells, and BCMA-CAR T cells, also in Figure 6c. Therefore, BsAb-CAR T have enhanced cell proliferation and augmented cell survival, most likely via the NKG2D signaling pathway.

**[0261]** To compare the survival and proliferation of unmodified T cells, EV T cells, BCMA CAR T cells, and BsAb-CAR T cells *in vivo*, Applicants injected (i.v.) these human cells into immunodeficient NSG mice (Figure 7A, upper). The background staining prior to i.v. injection of the human cells was also assessed on day -1 (Figure 7A, and Figure 18). On day +1 mice receiving each human T cell injection showed equal human CD3 expression, and the two CAR T cell populations were also identified by their $F(ab)_2$ expression. Moreover, the activation marker CD69 was detected on 97% of all four T cells populations isolated from the NSG mice (Figure 7A, and Figure 18). Interestingly, 14 days after the T cell infusions, only BsAb-CAR T cells group retained their high co-expression of CD3 and CD69 while there was substantial reduction of CD69 expression in the other three groups (Figure 7A, and Figure 18). Statistical analyses indicated that the day +14 percentage of both CD3 and $CD3^+CD69^+$ T cells in the BsAb-CAR T cell group were significantly higher than the other 3 groups (Figure 7B and 7C). Thirty-five days after the various transduced T cell injections, histograms showed that only the mice injected with BsAb-CAR T cells still

possessed high percentages of hCD3 T cells (Figure 7B). *In vivo* proliferation data shown that $Ki67^+CD69^+$ percentage was about 61.1% which was significantly higher than the three other groups that received non-transduced or transduced T cells (Figure 7a purple court panels, Figure 7c). Moreover, Applicants also determined the survival of BsAb-CAR T cells *in vivo* and observed that that the percentage of living (Annexin $V^-$Sytox $Blue^-$) BsAb-CAR T cells was approximately 87%, and the percentage of dead (Annexin $V^+$Sytox $Blue^+$) BsAb-CAR T cells was approximately 5%. Statistical analyses indicated BsAb-CAR T cells possessed a higher fraction of living cells and lower fraction of dead cells compared to the other three groups (Figure 7D). Taking together, BsAb-CAR T cells not only show enhanced the cell proliferation via NKG2D signaling pathway *in vitro*, but also demonstrate enhanced proliferation and survival *in vivo,* when compard to the other groups of injected cells (including BCMA CAR T cells).

**[0262]** *Improved recognition and killing of primary myeloma cells by BsAb-CAR T cells ex vivo*: To assess the clinical relevance of the BsAb-CAR T cells, Applicants investigated whether they could efficiently recognize and kill MM cells isolated from patients and enhance IFN-γ production *ex vivo*. Primary $CD138^+$ MM cells obtained from eight patients' bone marrow were isolated using positive magnetic selection, and flow cytometry was used to assess their surface expression of BCMA and CS1 (Figure 8A). Using a $^{51}Cr$ release assay performed in the absence of autologous PBMC, Applicants observed that MM cells from patients were highly resistant to EV-transduced T cell mediated lysis in all eight patients. Compared with BCMA CAR T cells or BsAb T cells, BsAb-CAR T cells showed significantly higher cytotoxicity in all eight patients that were tested, including patient 1 whose tumor cells had very low surface density expression of CS1. There is no significant difference in cytolytic activity between BsAb-CAR T cells and BsAb-BCMA seq. trans. T cells (Figure 8B). Applicants also measured IFN-γ after 24 hours in a similar co-culture assay; BsAb-CAR T cells also secreted significantly higher levels of IFN-γ than EV-transduced T cells, BsAb T cells, or BCMA-CAR T cells (Figure 8C). These findings demonstrate that BsAb-CAR T cells possess superb capacity to eradicate patient MM cells *ex vivo*.

**[0263]** *BsAb-CAR T cells inhibit MM tumor growth and prolong survival of tumor-bearing mice in an orthotopic xenograft MM model:* To further address the potential therapeutic application of BsAb-CAR T cells, Applicants examined their antitumor activity

in an MM.1S MM-engrafted NSG mouse model. Intravenous injection of MM.1S MM cells has been widely used to establish a mouse xenograft model of MM, because this can lead to bone marrow engraftment as well as consistent establishment of multifocal bone lytic lesions, which closely recapitulate human MM [43,44]. To facilitate monitoring of tumor growth, Applicants engineered MM.1S MM cells to express both GFP and firefly luciferase by retroviral infection, and used i.v. injection of 8 x $10^6$ of these MM cells to engraft NSG mice on day 0 as previously reported [30]. These mice were then infused on three occasions (day 10, day 17, and day 24) with i.v. saline or 1 x $10^7$ EV T cells, 1 x $10^7$ BsAb T cells, 1 x $10^7$ BCMA CAR T cells, 1 x $10^7$ BsAb-BCMA seq. trans. T cells, or 1 x $10^7$ BsAb-CAR T cells. For mice that survived up to day 80, Applicants collected peripheral blood lymphocytes and re-challenged the nice with 1 x $10^4$ MM.1S MM cells. Bioluminescence imaging was used to monitor the MM.1S MM growth and shows early disease progression up to day 31 in all six treatment groups (Figure 9A). Using an anti-$F(ab)_2$ antibody to identify BCMA CAR T cells, Applicants noted on day 80 that the percentages of BsAb-BCMA seq. trans. T cells and BsAb-CAR T cells were significantly higher in the blood of MM.1S MM mice than were BCMA CAR T (Figure 9B), while the total lymphocyte count in each mouse was similar (data not shown). Immunoblotting indicated the serum from day 80 MM.1S MM mice treated with BsAb-BCMA seq. trans. T cells or BsAb-CAR T cells still contained the secreted BsAb (not shown). By day 80, MM.1S MM mice treated with BsAb-BCMA seq. trans. T cells or with BsAb-CAR T cells had a significantly prolonged survival compared to similar mice treated with saline control, EV T cells or BsAb T cells. MM.1S MM mice treated with BCMA CAR T cells did slightly worse than MM.1S MM mice treated with BsAb-BCMA seq. trans. T cells or with BsAb-CAR T cells (Figure 9D). On day 140 (60 days after the first tumor re-challenge), all five of the MM.1S MM mice treated with BsAb-CAR T group survived (Figure 9D). These survival data following tumor re-challenge likely speak to the enhanced *in vivo* survival and enhanced *in vivo* proliferation of the BsAb-CAR T cells noted above in Figure 7. Overall, these results indicate that BsAb-CAR T cells are superior to the other non-transduced or transduced T cell populations in their ability to inhibit MM tumor growth and prolong survival of tumor-bearing mice in orthotopic xenograft MM model.

**[0264]** In order to determine the relevance of human non-transduced NKG2D+ lymphocytes in the orthotopic xenograft MM model (i.e., the *in vivo* counterpart to the *in vitro* experiment shown in Figure 3E), Applicants next examined the anti-tumor efficacy of

saline control, EV T cells, BCMA CAR T cells, or BsAb-CAR T cells in MM.1S MM-bearing NSG mice while co-injecting myeloid cell-depleted PBMC isolated from the same donor. The depletion of myeloid cells by sorting was undertaken to avoid GVHD (Figures 10A, 10B). The schema for injection of MM.1S MM cells, normal human lymphocytes, and various transduced T cells is shown in Figure 10A, as is the imaging documenting MM progression through day 37. Figures 10B and 10C illustrate the percentage human lymphocytes detected in the blood of these mice. With additional time under these conditions, Applicants found a dramatic difference between mice treated with BCMA CAR T cells (that do not secrete the BsAb; 0% survival by day 100) and mice treated with BsAb-CAR T cells (that do secrete the BsAb; 100% survival on day 140; Figure 10D). These data suggest that the more NKG2D+ immune cells that are involved, the better the efficacy of BsAb-CAR T cells will be for tumor eradication.

***Equivalents***

**[0265]** Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this technology belongs.

**[0266]** The present technology illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," *etc.* shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the present technology claimed.

**[0267]** Thus, it should be understood that the materials, methods, and examples provided here are representative of preferred aspects, are exemplary, and are not intended as limitations on the scope of the present technology.

**[0268]** The present technology has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the present technology. This includes the generic description of the present

technology with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

**[0269]** In addition, where features or aspects of the present technology are described in terms of Markush groups, those skilled in the art will recognize that the present technology is also thereby described in terms of any individual member or subgroup of members of the Markush group.

**[0270]** All publications, patent applications, patents, and other references mentioned herein are expressly incorporated by reference in their entirety, to the same extent as if each were incorporated by reference individually. In case of conflict, the present specification, including definitions, will control.

**[0271]** Other aspects are set forth within the following claims.

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**PARTIAL SEQUENCE LISTING**

**[0272]** Exemplary BCMA scFv:

**[0273]** *Anti-BCMA sequence 1 scFv heavy chain*

ATGGGATGGAGCTCTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCAC
CAGATTCAGCTGGTGCAGAGCGGCCCTGAGCTGAAGAAACCCGGCGAGACAGT
GAAGATCAGCTGCAAGGCCTCCGGCTACACCTTCCGGCACTACAGCATGAACTG
GGTGAAACAGGCCCCTGGCAAGGGCCTGAAGTGGATGGGCCGGATCAACACCG
AGAGCGGCGTGCCCATCTACGCCGACGACTTCAAGGGCAGATTCGCCTTCAGCG
TGGAAACCAGCGCCAGCACCGCCTACCTGGTGATCAACAACCTGAAGGACGAG
GATACCGCCAGCTACTTCTGCAGCAACGACTACCTGTACAGCCTGGACTTCTGG
GGCCAGGGCACCGCCCTGACCGTGTCCAGC

**[0274]** *Anti-BCMA sequence 1 scFv light chain*

GACATCGTGCTGACCCAGAGCCCCCCCAGCCTGGCCATGTCTCTGGGCAAGAGA
GCCACCATCAGCTGCCGGGCCAGCGAGAGCGTGACCATCCTGGGCAGCCACCT
GATCTACTGGTATCAGCAGAAGCCTGGCCAGCCCCCCACCCTGCTGATCCAGCT
GGCTAGCAATGTGCAGACCGGCGTGCCCGCCAGATTCAGCGGCAGCGGCAGCA
GAACCGACTTCACCCTGACCATCGACCCCGTGGAAGAGGACGACGTGGCCGTG
TACTACTGCCTGCAGAGCCGGACCATCCCCCGGACCTTTGGCGGAGGAACAAA
GCTGGAAATCAAG

**[0275]** *Anti-BCMA sequence 2 scFv heavy chain*

ATGGGATGGAGCTCTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCAC
CAGATTCAGCTGGTGCAGAGCGGCCCTGAGCTGAAGAAACCCGGCGAGACAGT
GAAGATCAGCTGCAAGGCCTCCGGCTACACCTTCACCGACTACAGCATCAACTG
GGTGAAAAGAGCCCCTGGCAAGGGCCTGAAGTGGATGGGCTGGATCAACACCG
AGACAAGAGAGCCCGCCTACGCCTACGACTTCCGGGGCAGATTCGCCTTCAGCC
TGGAAACCAGCGCCAGCACCGCCTACCTGCAGATCAACAACCTGAAGTACGAG

GACACCGCCACCTACTTTTGCGCCCTGGACTACAGCTACGCCATGGACTACTGG
GGCCAGGGCACCAGCGTGACCGTGTCCAGC

**[0276]** *Anti-BCMA sequence 2 scFv light chain*

GACATCGTGCTGACCCAGAGCCCCCCCAGCCTGGCCATGTCTCTGGGCAAGAGA
GCCACCATCAGCTGCCGGGCCAGCGAGAGCGTGACCATCCTGGGCAGCCACCT
GATCCACTGGTATCAGCAGAAGCCCGGCCAGCCCCCCACCCTGCTGATCCAGCT
CGCCAGCAATGTGCAGACCGGCGTGCCCGCCAGATTCAGCGGCAGCGGCAGCA
GAACCGACTTCACCCTGACCATCGACCCCGTGGAAGAGGACGACGTGGCCGTG
TACTACTGCCTGCAGAGCCGGACCATCCCCCGGACCTTTGGCGGAGGCACCAAA
CTGGAAATCAAG

**[0277]** *Anti-CS-1 scFv heavy chain DNA sequence*

AGCGTTACCG TGAGTACAGG CCAGGGCTGG TATGACATGG CACGTACAGC
CATCATGACC TCGCGCGCAT GTTACTACGT CGCGTCAGAT GAATCGACGC
CTTCCTCGCT GCAAATGTAT GCAACCTCCA GCAGCAAAGA TGTTACCCTG
ACCGCAAAGG ACAAGTTTAA ACAGAATTTG CGTACGGAGA GTGACTCCCC
GCACATCATG GGAATCTGGG AGTTGGGTCA GGGGCCTCGT CAGAAGGTAT
GGAACATGTG GTATACAACT TTTTCGTACG GCTCAGCAAA ATGCAGCTTG
AAAGTGTCGG CAGGTCCGCG CGTGCTGGAG GCCGGTCCGC AGCAGCTGCA
AGTCCAGTCT

**[0278]** *Anti-CS-1 scFv light chain DNA sequence*

AAACTTGAGT TGAAGACCGG TGCCGGCTTC ACCTTACCGA CCAGTTATCA
TCAACAATGC TATTACGTGG CCCTGGACGA AGCACAGGTG AATTCAATTA
CGTTTACGTT TGATACCGGC TCTGGCAGCG GTACATTTCG TGATCCCGTG
GGCACTTACC GCTATTCGGC GAGTTATATC TTGCTGAAAC CTTCCCAAGG
TCCGAAACAG CAGTACTGGG CGGTTGGCAC CATTGTAGAC CAATCAGCCA
AATGTACAAT CTCGGTTCGC GATGGTGTCA GTACGTCGAT GTCTAAGCAG
TCACAGACAA TGGTTATCGA T

**[0279]** *Anti-NKG2D heavy chain DNA sequence*

CAAGTGCAGC TGGTTGAATC CGGTGGCGGT CTGGTCAAGC CGGGCGGCTC
TTTGCGTCTG AGCTGTGCCG CGTCGGGTTT TACCTTCAGC TCTTATGGTA
TGCATTGGGT GCGTCAGGCG CCTGGCAAAG GTCTGGAGTG GGTTGCGTTC

ATCCGCTACG ATGGGTCTAA CAAATATTAT GCCGACTCAG TAAAAGGACG CTTCACTATT AGCCGCGACA ATAGCAAAAA TACCCTGTAC CTGCAAATGA ATAGCCTGCG CGCCGAAGAT ACCGCCGTTT ACTATTGCGC TAAAGATCGT GGCCTGGGTG ATGGTACGTA CTTCGATTAC TGGGGTCAGG GCACCACCGT TACCGTTAGT TCA

**[0280]** *Anti-NKG2D light chain DNA sequence*

CAGTCAGCGC TTACGCAGCC GGCGTCGGTG TCGGGTTCCC CGGGTCAGTC GATCACGATC AGCTGTAGTG GGAGCAGCTC CAACATCGGT AACAACGCAG TGAACTGGTA TCAGCAACTG CCGGGAAAAG CGCCGAAACT GCTGATTTAC TATGATGATT TGCTGCCAAG TGGAGTTAGT GACCGCTTTT CCGGCAGTAA ATCGGGTACC TCGGCTTTTC TGGCTATTTC GGGTCTCCAG AGCGAGGATG AAGCTGATTA TTATTGCGCC GCATGGGATG ATAGCTTAAA TGGCCCAGTT TTTGGCGGCG GTACTAAACT GACCGTGCTG

**[0281]** *Linker*

GGCGGTGGCGGTTCTGGTGGCGGTGGCTCCGGCGGTGGCGGTTCT

**[0282]** *Anti-NKG2D Heavy Chain*

CAAGTGCAGCTGGTTGAATCCGGTGGCGGTCTGGTCAAGCCGGGCGGCTCTTTG CGTCTGAGCTGTGCCGCGTCGGGTTTTACCTTCAGCTCTTATGGTATGCATTGGG TGCGTCAGGCGCCTGGCAAAGGTCTGGAGTGGGTTGCGTTCATCCGCTACGATG GGTCTAACAAATATTATGCCGACTCAGTAAAAGGACGCTTCACTATTAGCCGCG ACAATAGCAAAAATACCCTGTACCTGCAAATGAATAGCCTGCGCGCCGAAGAT ACCGCCGTTTACTATTGCGCTAAAGATCGTGGCCTGGGTGATGGTACGTACTTC GATTACTGGGGTCAGGGCACCACCGTTACCGTTAGTTCAGGTGGGGGCGGCTCT

**[0283]** *Anti-NKG2D Light Chain*

CAGCGCTTACGCAGCCGGCGTCGGTGTCGGGTTCCCCGGGTCAGTCGATCACGA TCAGCTGTAGTGGGAGCAGCTCCAACATCGGTAACAACGCAGTGAACTGGTAT CAGCAACTGCCGGGAAAAGCGCCGAAACTGCTGATTTACTATGATGATTTGCTG CCAAGTGGAGTTAGTGACCGCTTTTCCGGCAGTAAATCGGGTACCTCGGCTTTT CTGGCTATTTCGGGTCTCCAGAGCGAGGATGAAGCTGATTATTATTGCGCCGCA TGGGATGATAGCTTAAATGGCCCAGTTTTTGGCGGCGGTACTAAACTGACCGTG CTG

**[0284]** *HMA Sequences*

CCGAGCGGCCAGGCGGGCGCGGCGGCATCGGAGTCCCTGTTTGTGTCAAATCA
CGCCTAC

**[0285]** *Anti-CS1 Heavy Chain*

CTCCGTGACGGTGTCGACGGGCCAAGGATGGTACGATATGGCACGGACCGCGA
TTATGACATCGCGGGCGTGCTATTACGTGGCCAGCGATGAGTCGACCCCTTCCT
CTCTGCAAATGTATGCCACCTCCTCTTCAAAAGACGTGACTCTGACTGCGAAAG
ACAAATTTAAACAGAATCTGCGCACCGAAAGCGATAGCCCACATATCATGGGC
ATCTGGGAACTGGGCCAGGGCCCCCGCCAGAAAGTGTGGAACATGTGGTACAC
CACCTTCAGCTATGGTTCGGCCAAATGTTCCCTGAAGGTATCAGCCGGCCCGCG
CGTTCTTGAGGCGGGTCCGCAGCAGCTGCAGGTACAGAGC

**[0286]** *Anti-CS1 Light Chain*

AAACTGGAACTCAAGACGGGTGCGGGATTTACCCTCCCTACGAGCTATCACCAG
CAGTGCTATTACGTGGCGCTTGACGAAGCGCAGGTGAACTCTATTACCTTTACC
TTTGATACAGGATCAGGCAGCGGTACGTTCCGTGATCCGGTAGGTACGTACCGG
TATAGTGCAAGCTATATCCTTCTGAAACCTTCTCAGGGTCCGAAACAGCAGTAC
TGGGCGGTGGGAACGATCGTGGACCAGTCTGCCAAATGTACAATTTCAGTTCGC
GACGGAGTTAGCACCTCCATGAGCAAGCAGTCCCAAACCATGGTGATTGACTCT

**[0287]** *Anti-BCMA Heavy Chain Sequence 2*

CAGATTCAGCTGGTGCAGAGCGGCCCTGAGCTGAAGAAACCCGGCGAGACAGT
GAAGATCAGCTGCAAGGCCTCCGGCTACACCTTCACCGACTACAGCATCAACTG
GGTGAAAAGAGCCCCTGGCAAGGGCCTGAAGTGGATGGGCTGGATCAACACCG
AGACAAGAGAGCCCGCCTACGCCTACGACTTCCGGGGCAGATTCGCCTTCAGCC
TGGAAACCAGCGCCAGCACCGCCTACCTGCAGATCAACAACCTGAAGTACGAG
GACACCGCCACCTACTTTTGCGCCCTGGACTACAGCTACGCCATGGACTACTGG
GGCCAGGGCACCAGCGTGACCGTGTCCAGC

**[0288]** *Anti-BCMA Light Chain Sequence 2*

GACATCGTGCTGACCCAGAGCCCCCCCAGCCTGGCCATGTCTCTGGGCAAGAGA
GCCACCATCAGCTGCCGGGCCAGCGAGAGCGTGACCATCCTGGGCAGCCACCT

GATCCACTGGTATCAGCAGAAGCCCGGCCAGCCCCCCACCCTGCTGATCCAGCT
CGCCAGCAATGTGCAGACCGGCGTGCCCGCCAGATTCAGCGGCAGCGGCAGCA
GAACCGACTTCACCCTGACCATCGACCCCGTGGAAGAGGACGACGTGGCCGTG
TACTACTGCCTGCAGAGCCGGACCATCCCCCGGACCTTTGGCGGAGGCACCAAA
CTGGAAATCAAG

**WHAT IS CLAIMED IS:**

1. A vector comprising:

a polynucleotide encoding a chimeric antigen receptor (CAR) comprising: (a) an antigen binding domain of a cancer or tumor targeting antibody; (b) a hinge domain; (c) a transmembrane domain; (d) and an intracellular domain; and

a polynucleotide encoding a bispecific antibody comprising an antigen binding domain that recognizes and binds NKG2D.

2. The vector of claim 1, wherein the CAR comprises: (a) an antigen binding domain of a a cancer or tumor targeting antibody; (b) a CD8 α hinge domain; (c) a CD8 α transmembrane domain; (d) a CD28 costimulatory signaling region and/or a 4-1BB costimulatory signaling region; and (e) a CD3 zeta signaling domain.

3. The vector of claims 1 or 2, wherein the cancer or tumor targeting antibody targets B-cell maturation antigen (BCMA) and/or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof.

4. The vector of any one of claims 1 to 3, wherein the bispecific antibody comprises a ligand of NKG2D or an anti-NKG2D scFv and an antigen binding domain of an anti-SLAMF7 antibody (also known as CS1 or CD319), and/or an equivalent each thereof.

5. The vector of any one of claims 1 to 3, wherein the bispecific antibody comprises CDR regions of an antibody to NKG2D and an antigen binding domain of an anti-SLAMF7 antibody (also known as CS1 or CD319), and/or an equivalent of each thereof.

6. The vector of claim 5, wherein the bispecific antibody comprises the heavy chain and light chain variable region of an antibody to NKG2D and an antigen binding domain of an anti-SLAMF7 antibody (also known as CS1 or CD319), and/or an equivalent of each thereof.

7. The vector of claim 5 or 6, wherein the bispecific antibody comprises a single chain variable fragment (scFV) derived from an antibody to NKG2D, optionally, a single chain variable fragment (scFv) derived from an anti-SALMF7 antibody (also known as CS1 of CD319), and/or an equivalent each thereof.

8. The vector of any one of claims 1 to 7, wherein the vector is a plasmid, and optionally a promoter to regulate expressson of the polynucleotide.

9. The vector of any one of claims 1 to 7, wherein the vector is a viral vector selected from the group of a retroviral vector, a lentiviral vector, an adenoviral vector, and an adeno-associated viral vector, and optionally a promoter to regulate expressson of the polynucleotide.

10. An isolated cell comprising the vector of any one of claims 1 to 9.

11. The isolated cell of claim 10, wherein the cell is a prokaryotic cell or a eukaryotic cell.

12. The isolated cell of claim 11, wherein the cell is a eukaryotic cell.

13. The isolated of claim 12, wherein the eukaryotic cell is selected from an animal cell, a mammalian cell, a bovine cell, a feline cell, a canine cell, a murine cell, an equine cell or a human cell.

14. The isolated cell of claim 12 or 13, wherein the eukaryotic cell is an immune cell, optionally a T-cell, a B cell, a NK cell, a dendritic cell, a myeloid cell, a monocyte, or a macrophage.

15. The isolated cell of any one of claims 10 to 14, wherein the isolated cell expresses the CAR and secretes the bispecific antibody.

16. A composition comprising the vector of any one of claims 1 to 9 and/or the isolated cell of any one of claims 10 to 15, and, optionally, a pharmaceutically acceptable carrier.

17. An isolated complex comprising an isolated cell comprising of any one of claims 10 to 15 bound to a cell expressing a cancer or tumor antigen, optionally, wherein the cancer or tumor antigen is NKG2D and/or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof.

18. An isolated complex comprising the isolated cell of any one of claims 10 to 15 bound to a cancer or tumor antigen or a fragment thereof, optionally, wherein the cancer or tumor antigen is BCMA or SLAMF7 (also known as CS1 or CD319), and/or an equivalent of each thereof.

19. A method of producing a CAR expressing cell comprising transducing an isolated cell with vector of any one of claims 1 to 9.

20. The method of claim 18, wherein the isolated cells are selected from a group consisting of T-cells, B cells, NK cells, dendritic cells, myeloid cells, monocytes, or macrophages.

21. A method of inhibiting the growth of a cancer cell or tumor tumor expressing a cancer or tumor antigen, comprising contacting the cancer cell or tumor with the isolated cell of any one of claims 10 to 15.

22. The method of claim 21, wherein the contacting is *in vitro* or *in vivo*.

23. The method of claim 22, wherein the contacting is in vivo and the isolated cells are autologous or allogeneic to a subject being treated.

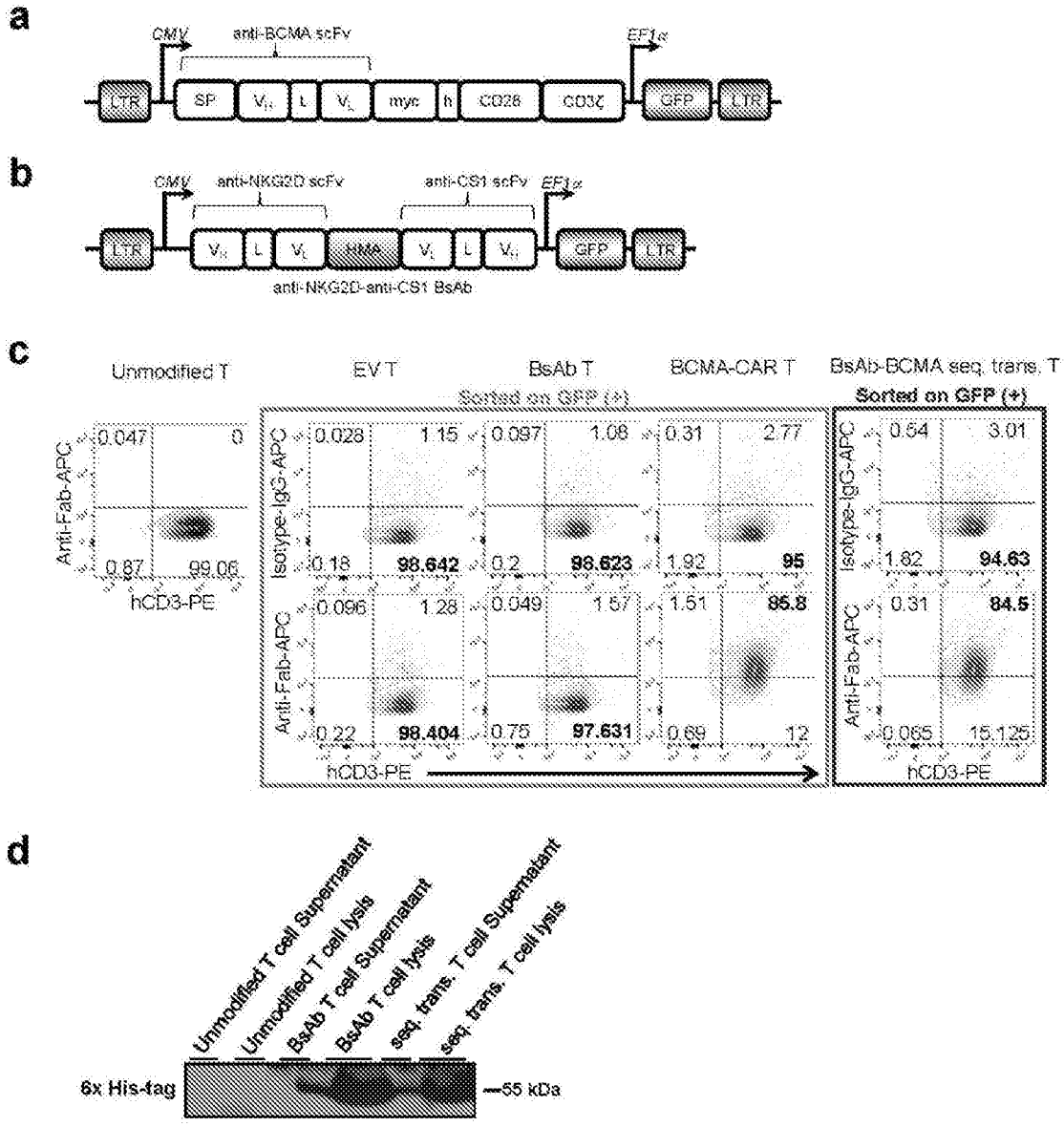
24. The method of claim 21, wherein the contacting is *in vivo* and the isolated cells are allogenic to a subject being treated.

25. The method of claim 23 or 24, further comprising administering to the subject an effective amount of a cytoreductive therapy or chemotherapy or therapy that upregulates the expression of a target antigen.

26. The method of claim 25, wherein the cytoreductive therapy comprises chemotherapy, cryotherapy, hyperthermia, targeted therapy, and/or radiation therapy.

27. The method of any one of claims 21 to 26, wherein the subject is a mammal, a canine, a feline, an equine, a murine or a human patient.

28. A kit comprising a composition as disclosed herein and optionally, instructions for use.


a
CMV
anti-BCMA scFv
EF1α
LTR
SP
L
myc
h
CD28
CD3ζ
GFP
LTR
b
CMV
anti-NKG2D scFv
anti-CS1 scFv
EF1α
LTR
L
HMA
L
GFP
LTR
anti-NKG2D-anti-CS1 BsAb
c
Unmodified T
EV T
BsAb T
BCMA-CAR T
BsAb-BCMA seq. trans. T
Sorted on GFP (+)
Sorted on GFP (+)
Anti-Fab-APC
Isotype-IgG-APC
hCD3-PE
0.047
0
0.87
99.06
0.028
1.15
0.18
98.642
0.097
1.08
0.2
98.623
0.31
2.77
1.92
95
0.54
3.01
1.82
94.63
0.096
1.28
0.22
98.404
0.049
1.57
0.75
97.631
1.51
96.8
0.69
12
0.31
84.5
0.065
15.125
d
Unmodified T cell Supernatant
Unmodified T cell lysis
BsAb T cell Supernatant
BsAb T cell lysis
seq. trans. T cell Supernatant
seq. trans. T cell lysis
6x His-tag
55 kDa

**FIGS. 1A-1D**

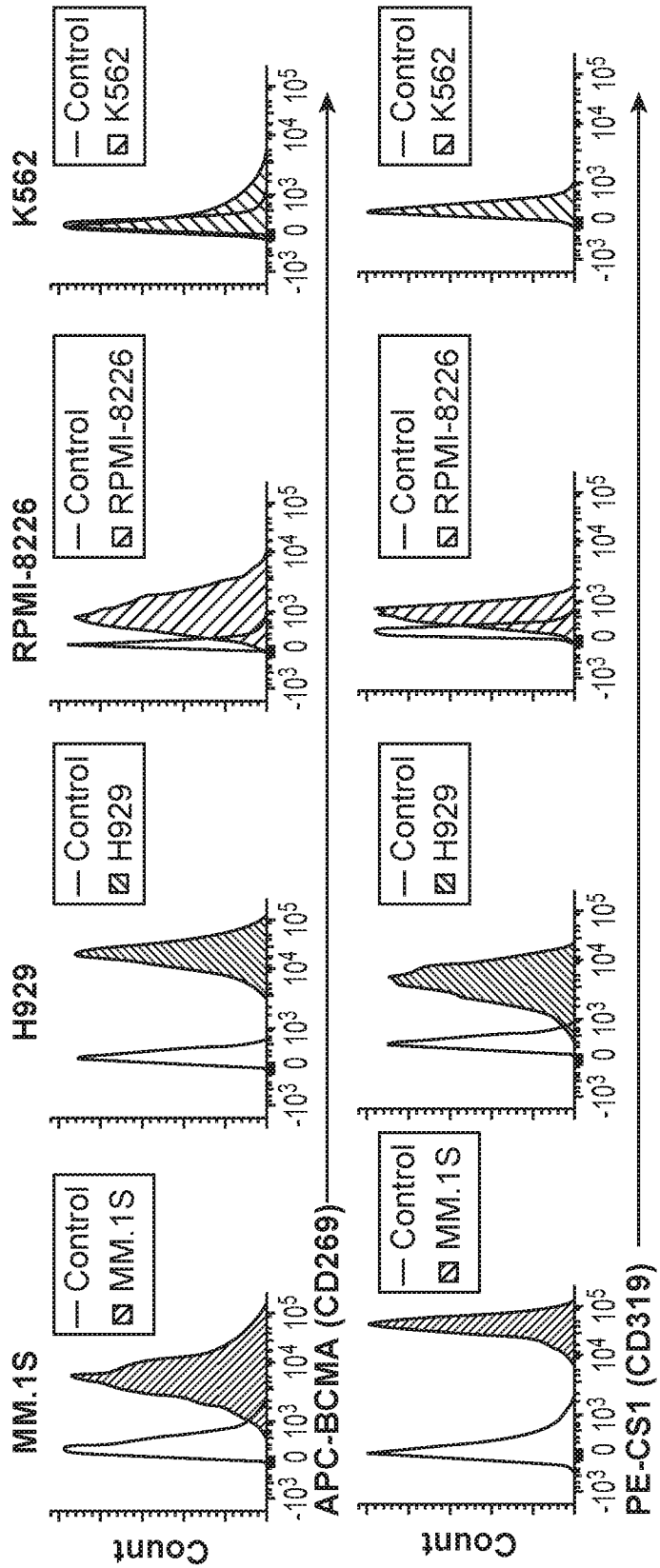

MM.1S
H929
RPMI-8226
K562
Control
MM.1S
H929
RPMI-8226
K562
Count
APC-BCMA (CD269)
PE-CS1 (CD319)

FIG. 2A

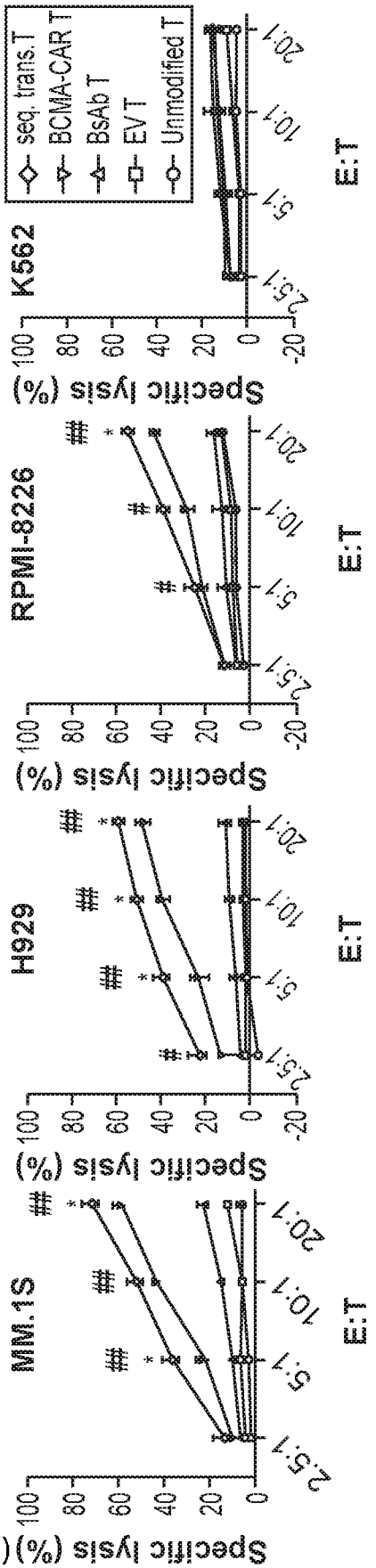

MM.1S
H929
RPMI-8226
K562
Specific lysis (%)
E:T
2.5:1
5:1
10:1
20:1
seq. trans.T
BCMA-CAR T
BsAb T
EV T
Unmodified T

FIG. 2B

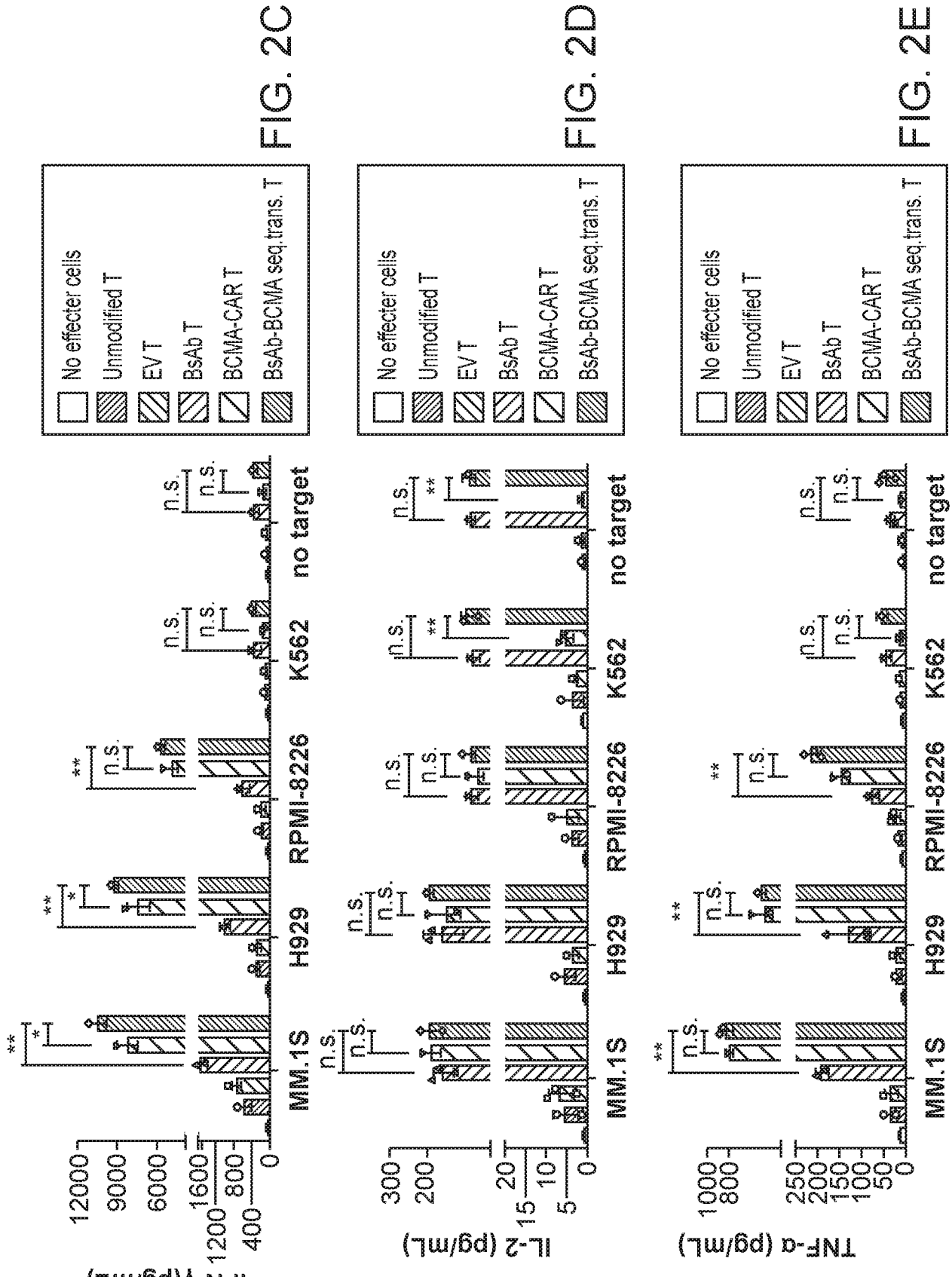



FIG. 2C

FIG. 2D

FIG. 2E

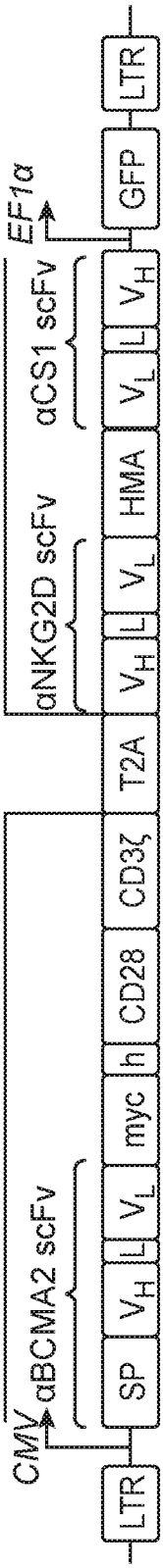



FIG. 3A

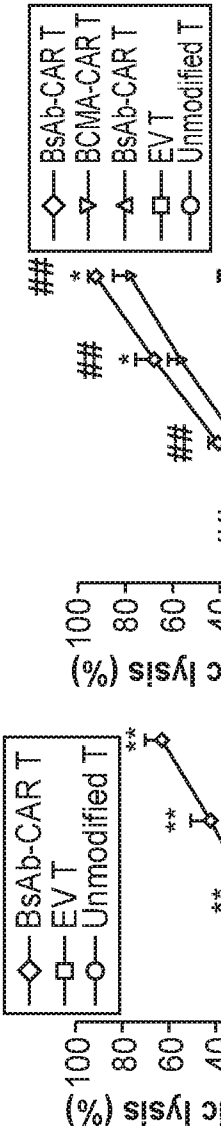






FIG. 3C

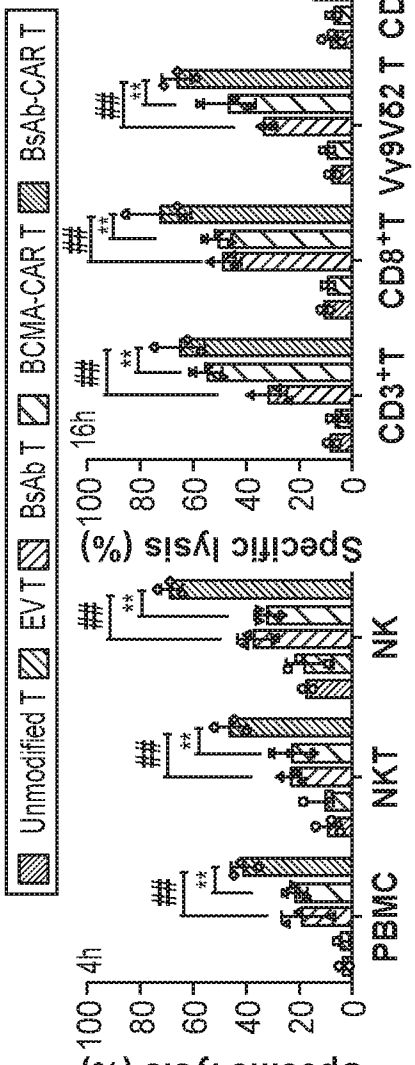
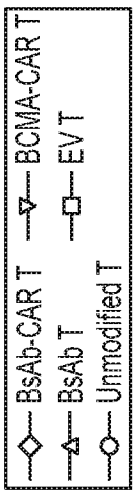



FIG. 3D


FIG. 3B



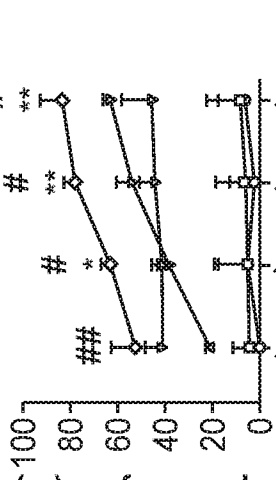




FIG. 3E

FIG. 3F

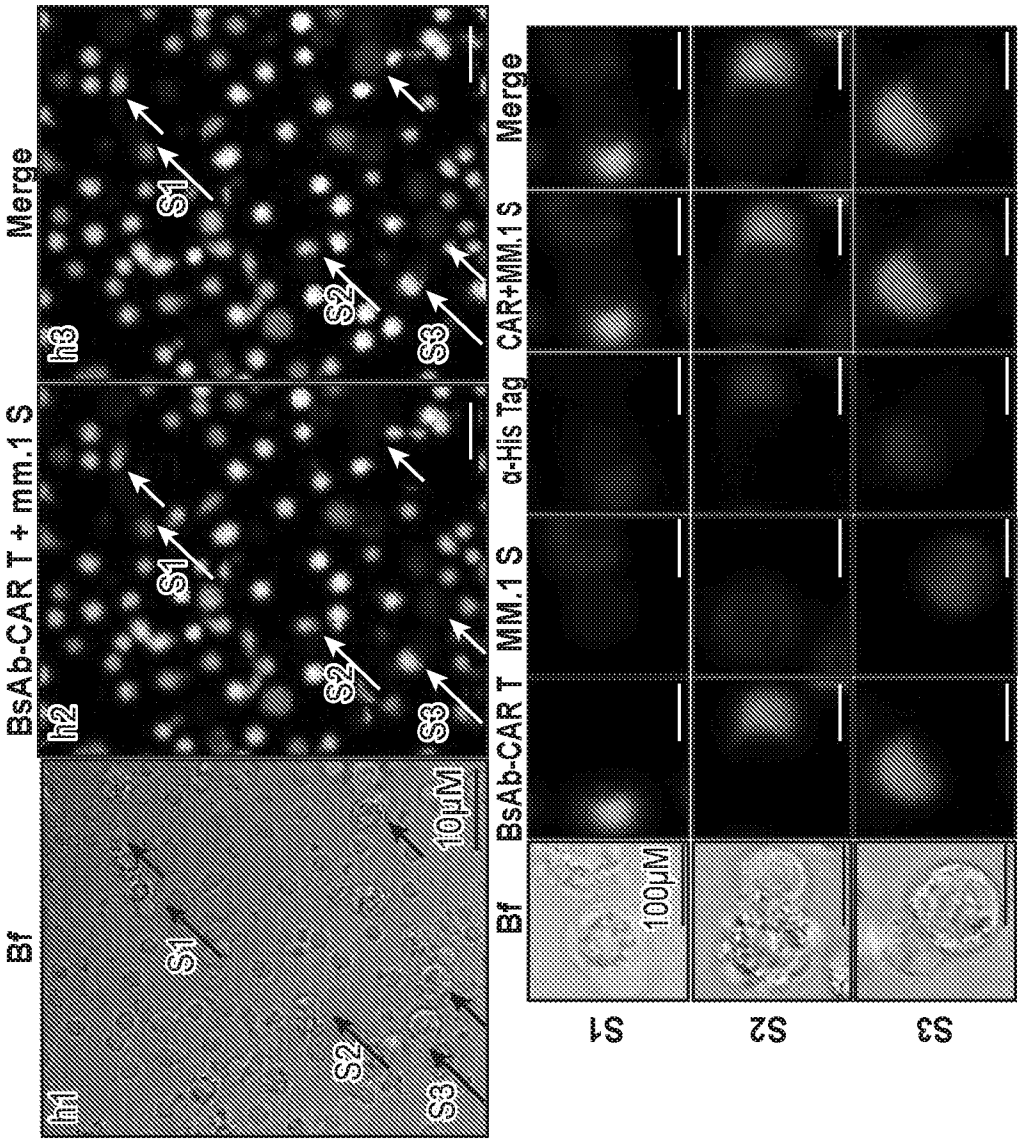

Bf
BsAb-CAR T + mm.1 S
Merge
h1
h2
h3
S1
S2
S3
10μM
Bf
BsAb-CAR T
MM.1 S
α-His Tag
CAR+MM.1 S
Merge
100μM

FIG. 3H

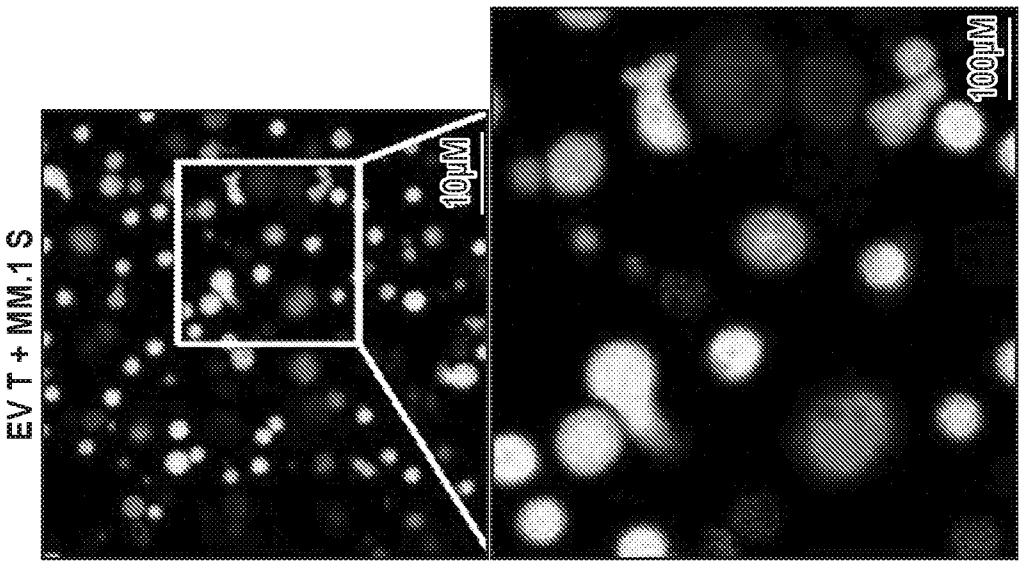

EV T + MM.1 S
10μM
100μM

FIG. 3G

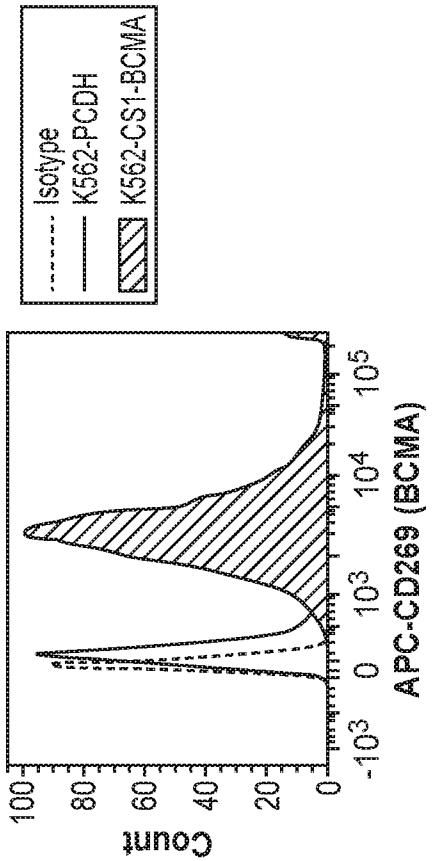



FIG. 4A

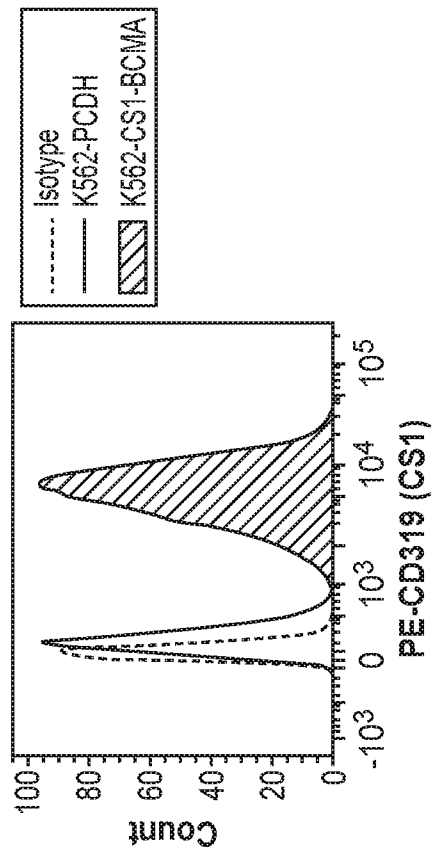



FIG. 4B

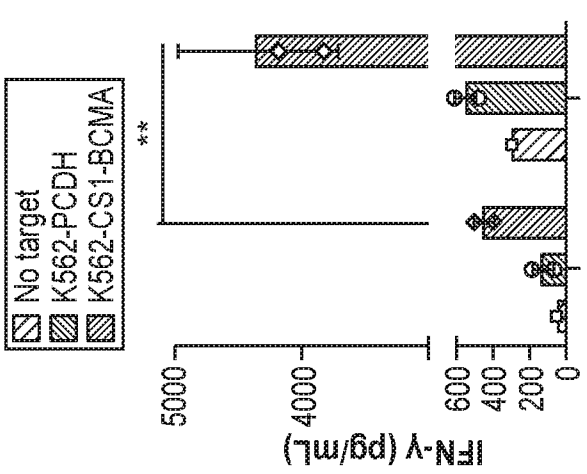



FIG. 4C

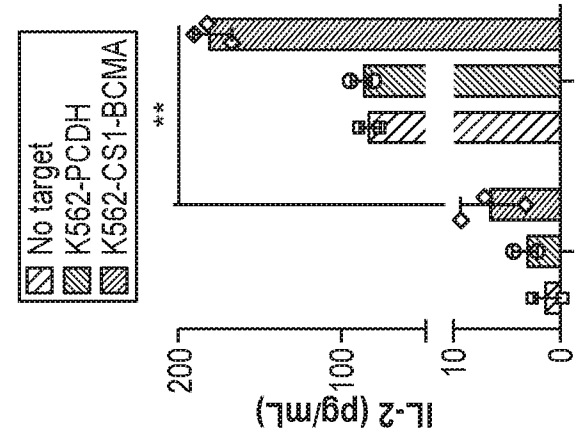



FIG. 4D

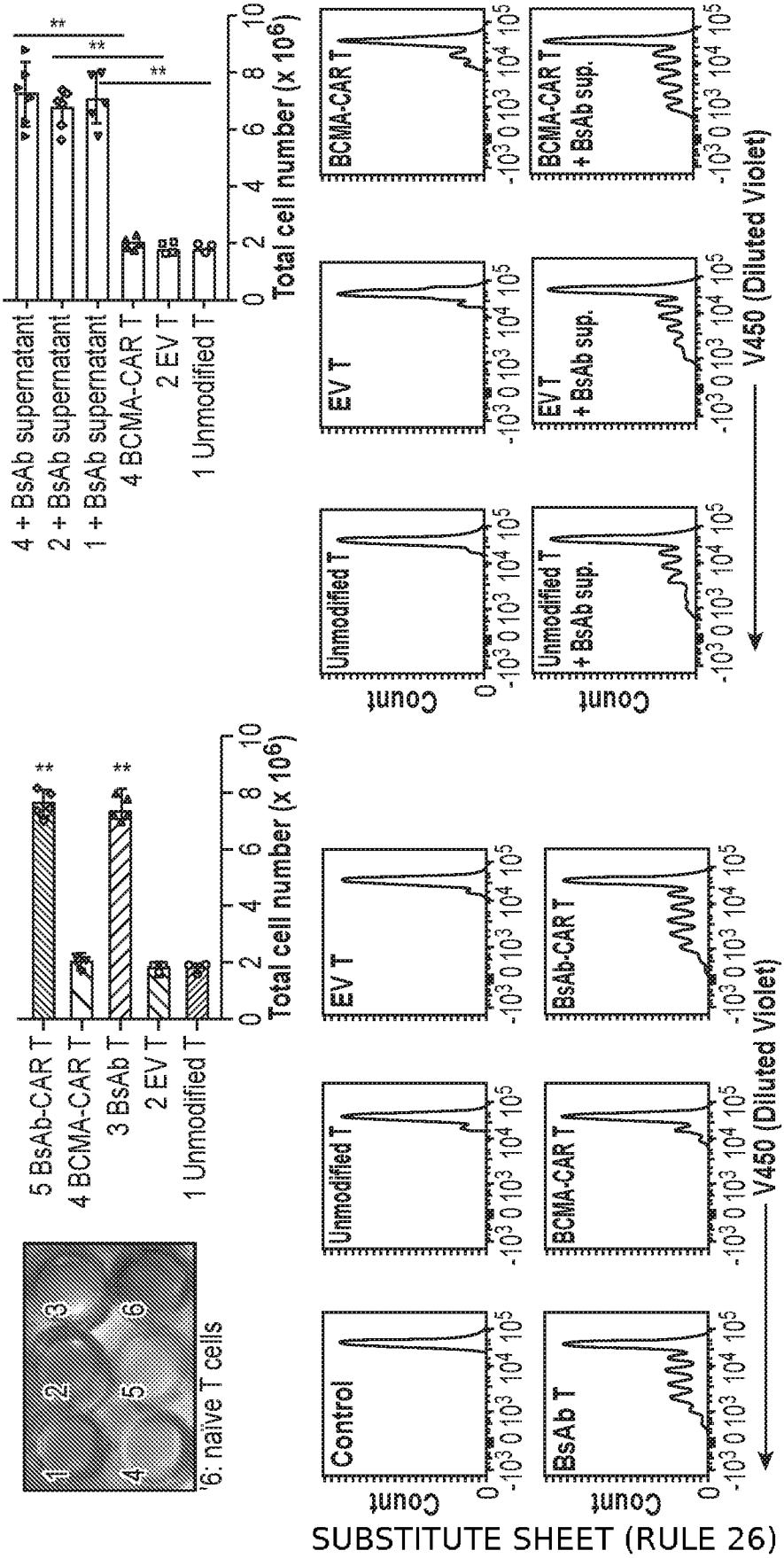



FIG. 5A

FIG. 5B

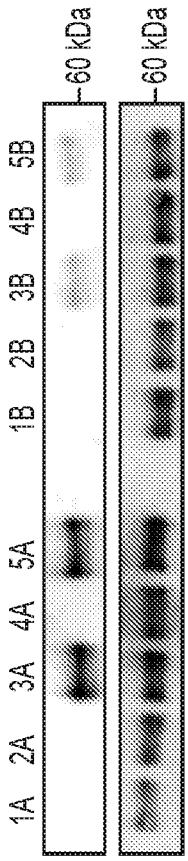



FIG. 5D




FIG. 5C

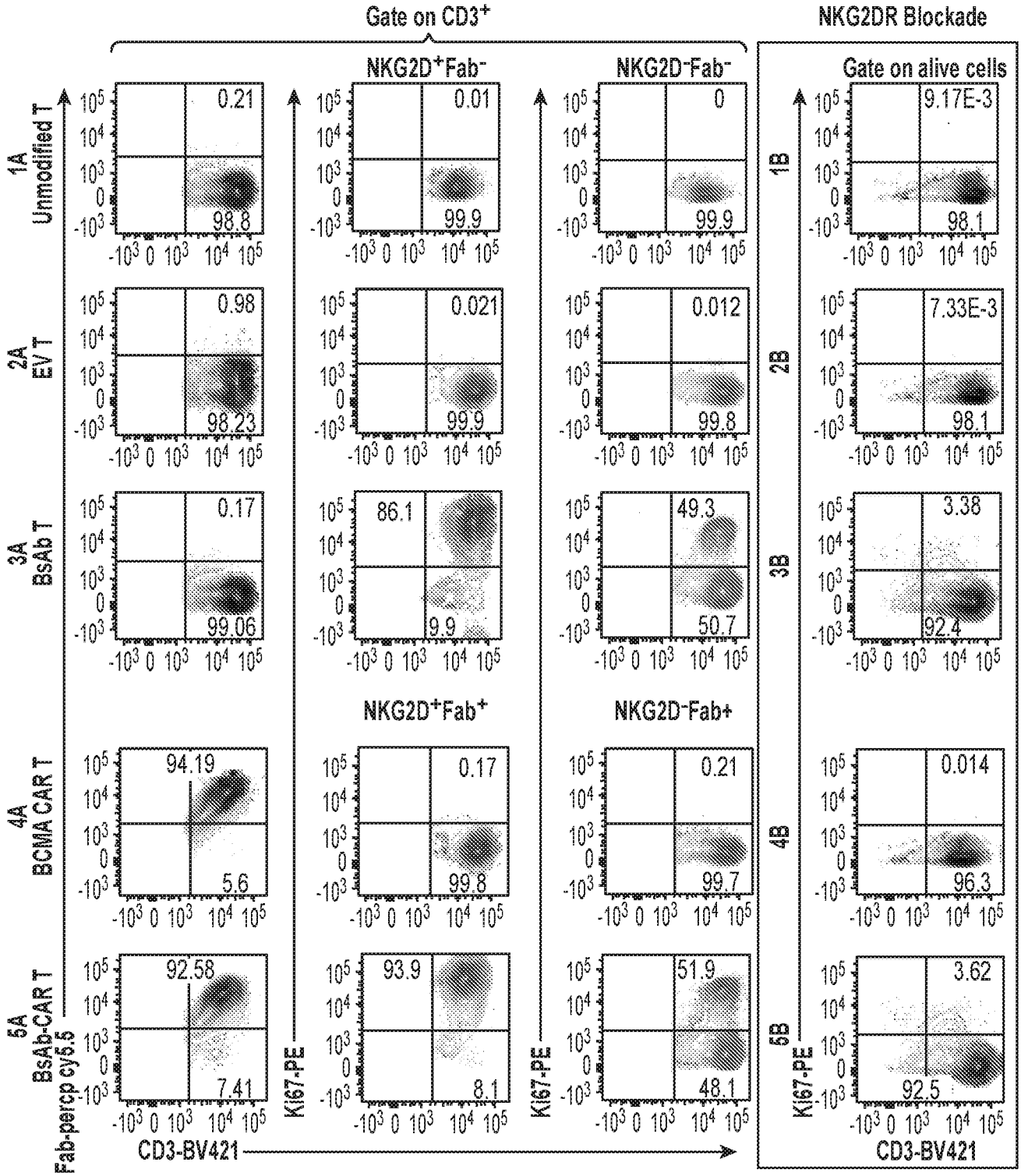

Gate on CD3+
NKG2DR Blockade
NKG2D+Fab-
NKG2D-Fab-
Gate on alive cells
1A Unmodified T
2A EV T
3A BsAb T
4A BCMA CAR T
5A BsAb-CAR T
1B
2B
3B
4B
5B
0.21
0.01
0
9.17E-3
98.8
99.9
99.9
98.1
0.98
0.021
0.012
7.33E-3
98.23
99.9
99.8
98.1
0.17
86.1
49.3
3.38
99.06
9.9
50.7
92.4
NKG2D+Fab+
NKG2D-Fab+
94.19
0.17
0.21
0.014
5.6
99.8
99.7
96.3
92.58
93.9
51.9
3.62
7.41
8.1
48.1
92.5
Fab-percp cy5.5
CD3-BV421
Ki67-PE
Ki67-PE
CD3-BV421

FIG. 5E

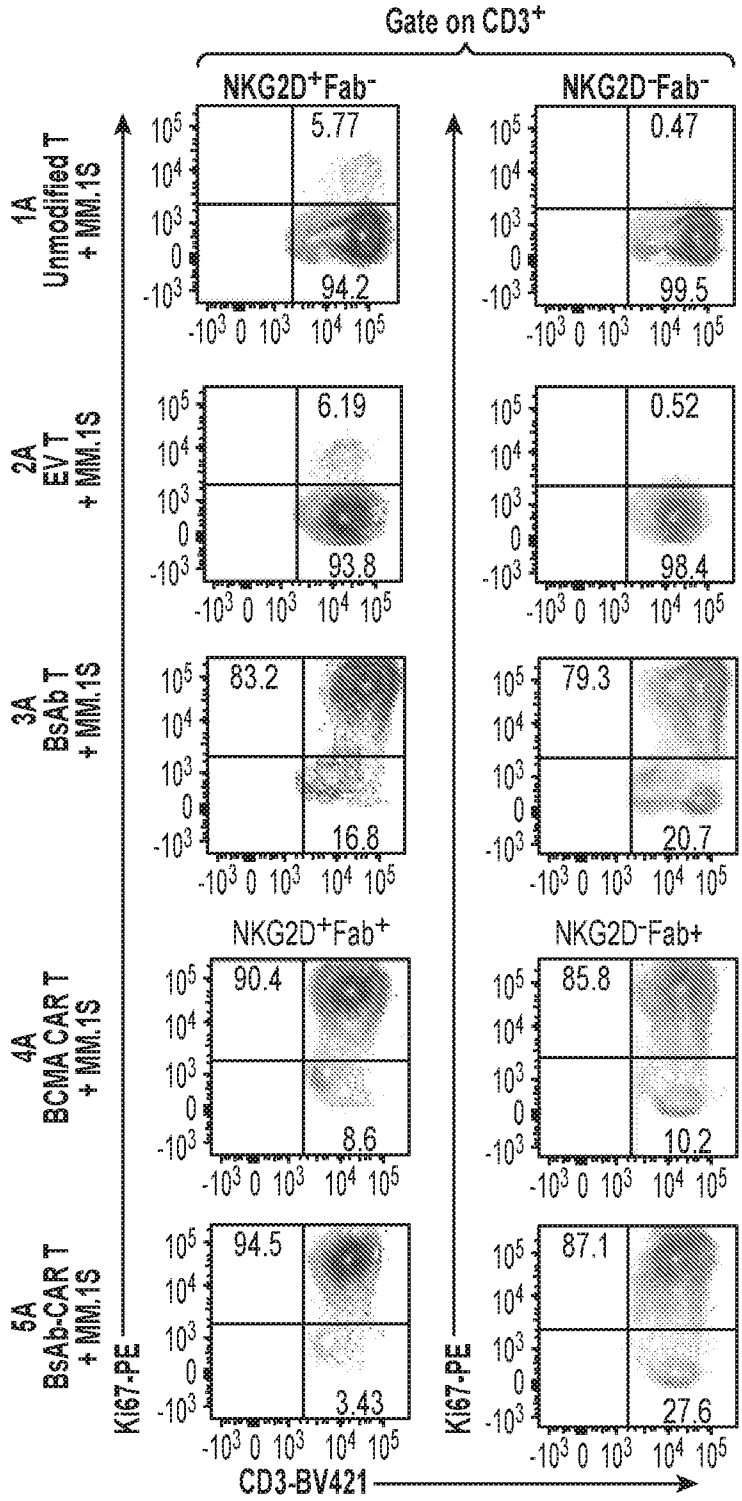
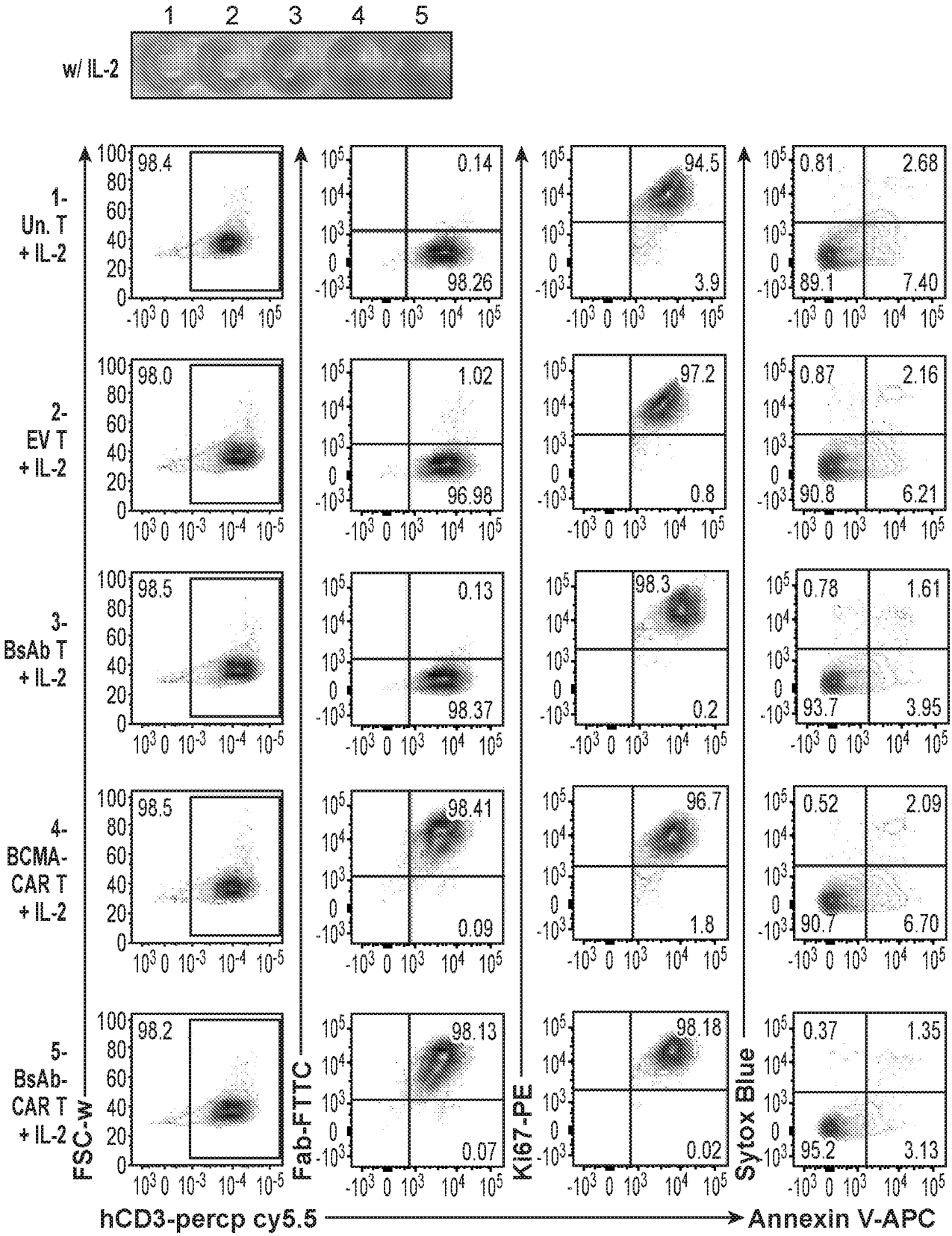

Gate on CD3+
NKG2D+Fab-
NKG2D-Fab-
1A Unmodified T + MM.1S
2A EV T + MM.1S
3A BsAb T + MM.1S
4A BCMA CAR T + MM.1S
5A BsAb-CAR T + MM.1S
5.77
94.2
0.47
99.5
6.19
93.8
0.52
98.4
83.2
16.8
79.3
20.7
NKG2D+Fab+
NKG2D-Fab+
90.4
8.6
85.8
10.2
94.5
3.43
87.1
27.6
Ki67-PE
CD3-BV421

FIG. 5E (Cont.)




SUBSTITUTE SHEET (RULE 26) FIG. 6A

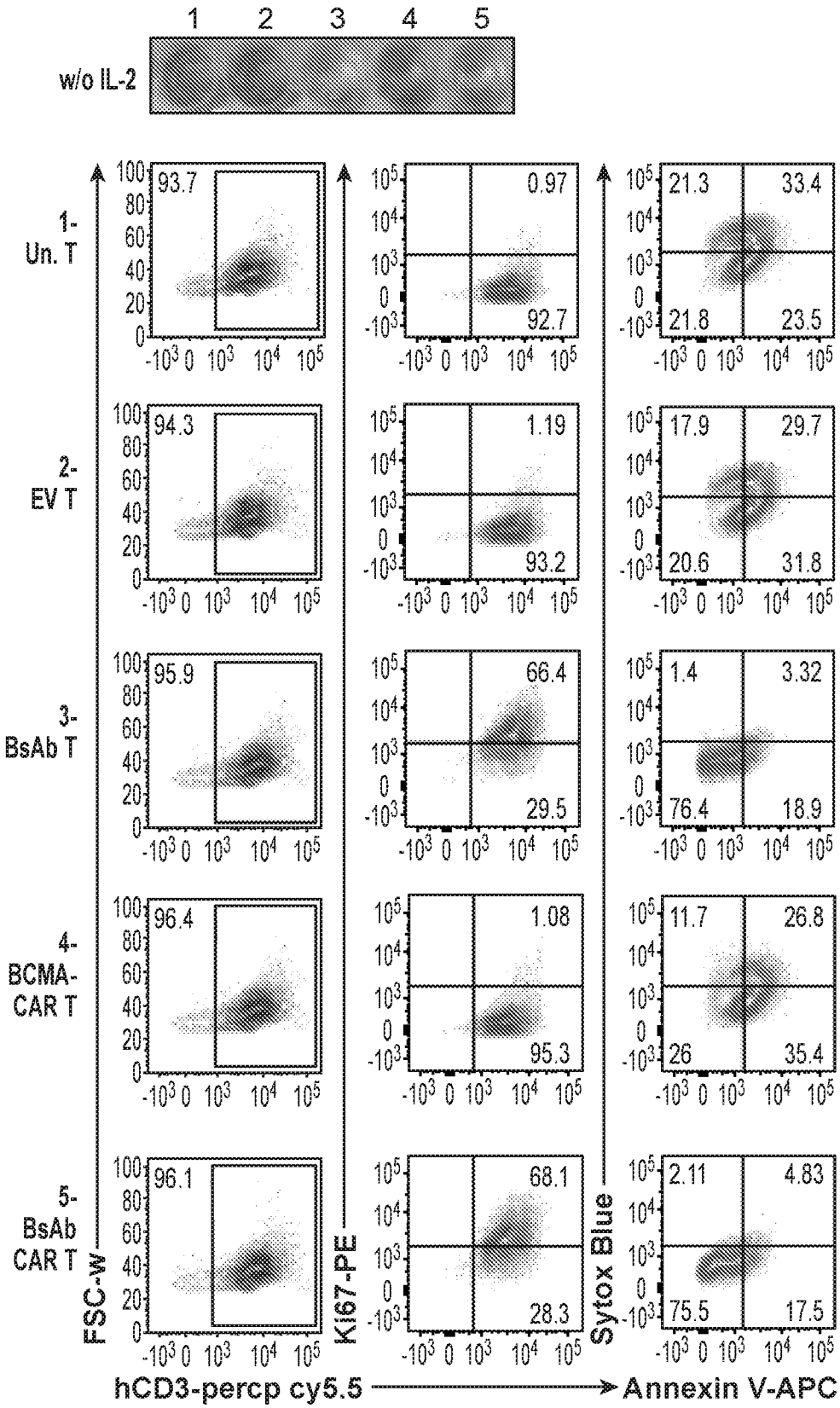

1 2 3 4 5
w/o IL-2
1- Un. T
2- EV T
3- BsAb T
4- BCMA- CAR T
5- BsAb CAR T
93.7
0.97
21.3
33.4
21.8
23.5
94.3
1.19
17.9
29.7
93.2
20.6
31.8
95.9
66.4
1.4
3.32
29.5
76.4
18.9
96.4
1.08
11.7
26.8
95.3
26
35.4
96.1
68.1
2.11
4.83
28.3
75.5
17.5
FSC-W
Ki67-PE
Sytox Blue
hCD3-percp cy5.5
Annexin V-APC

FIG. 6B

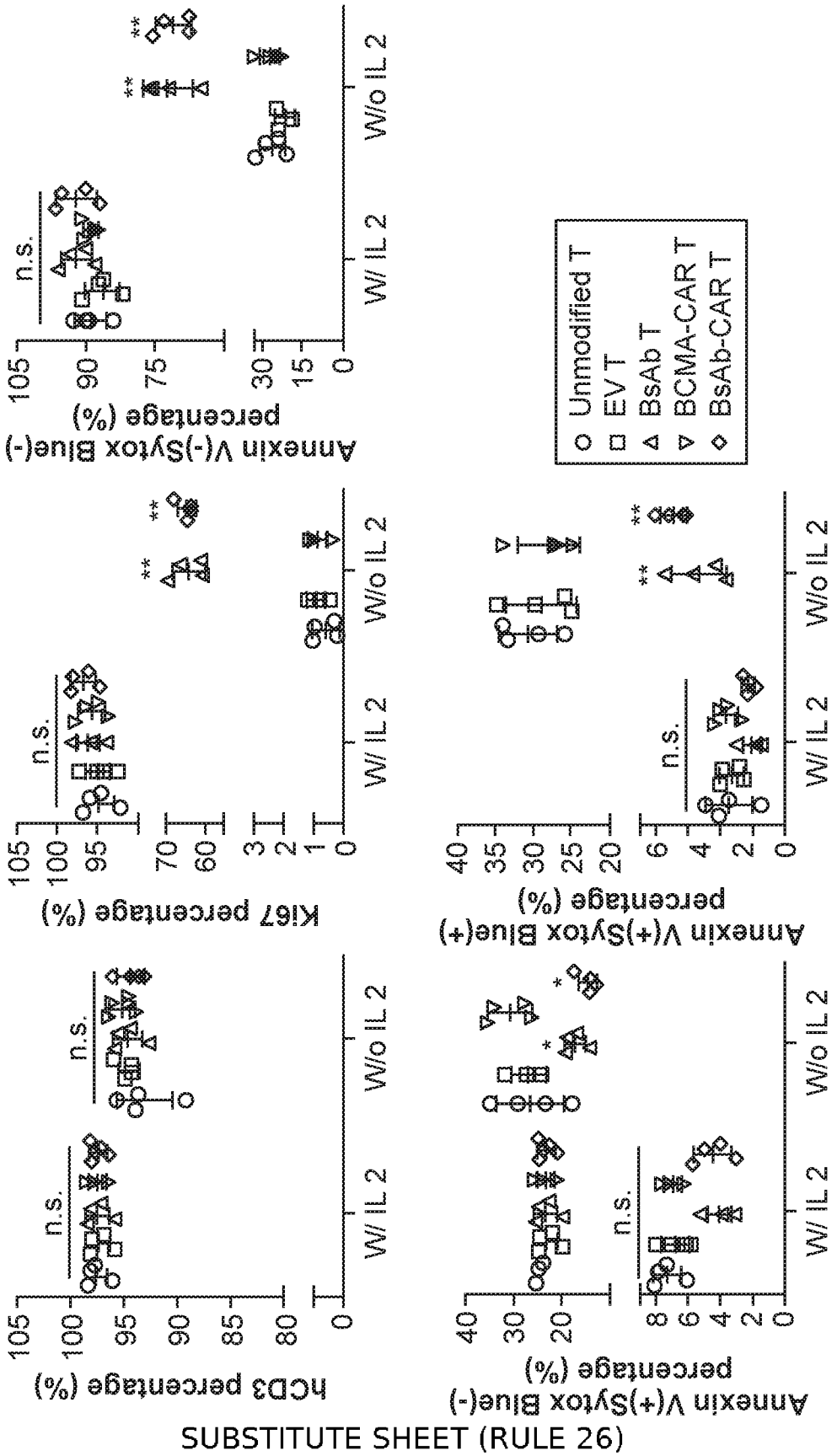

Annexin V(-)Sytox Blue(-) percentage (%)
Annexin V(+)Sytox Blue(-) percentage (%)
Annexin V(+)Sytox Blue(+) percentage (%)
Ki67 percentage (%)
hCD3 percentage (%)
n.s.
W/ IL 2
W/o IL 2
Unmodified T
EV T
BsAb T
BCMA-CAR T
BsAb-CAR T

FIG. 6C

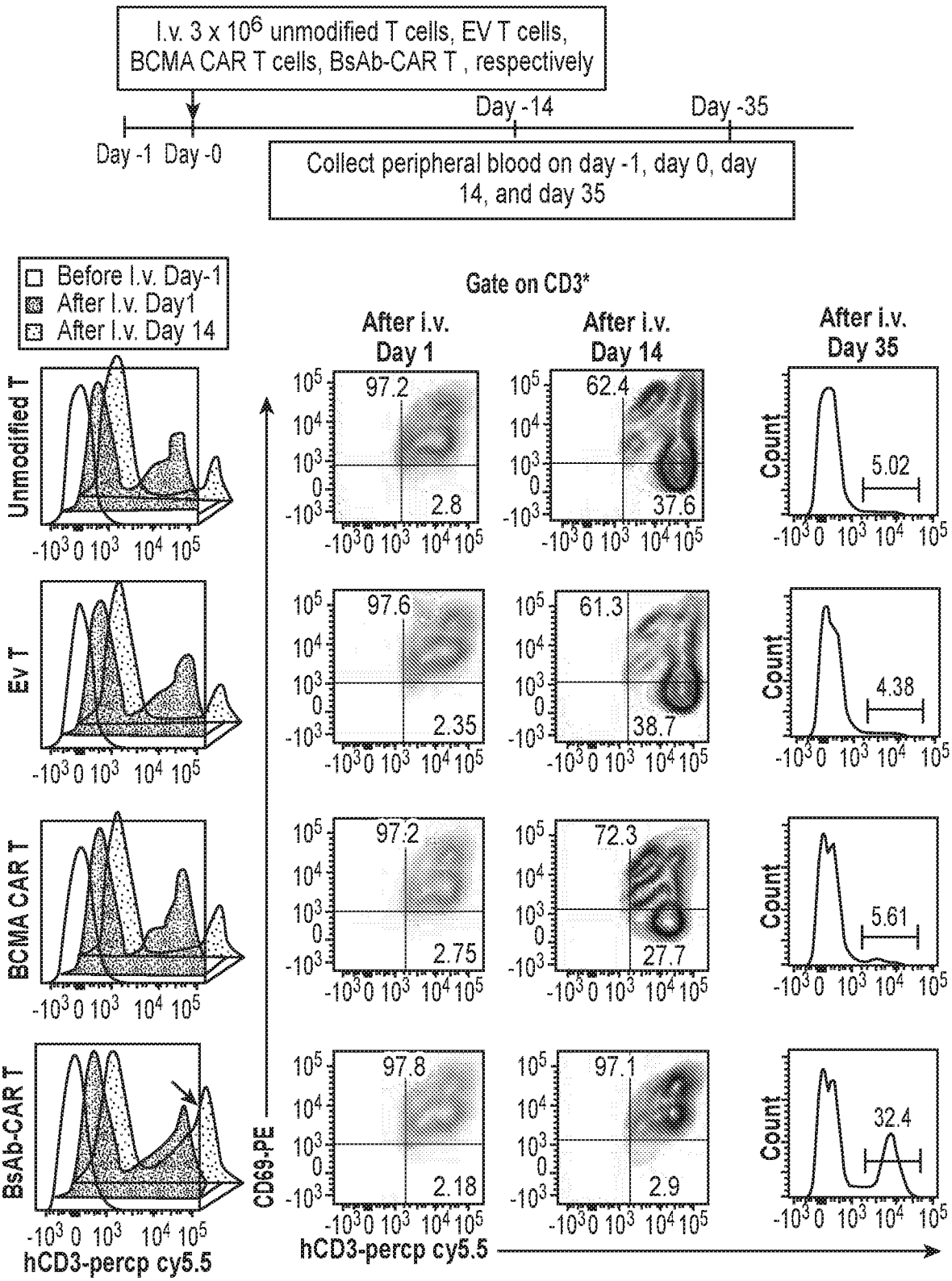

I.v. 3 x 10^6 unmodified T cells, EV T cells, BCMA CAR T cells, BsAb-CAR T , respectively
Day -1 Day -0
Day -14
Day -35
Collect peripheral blood on day -1, day 0, day 14, and day 35
Before I.v. Day-1
After I.v. Day1
After I.v. Day 14
Gate on CD3*
After i.v. Day 1
After i.v. Day 14
After i.v. Day 35
Unmodified T
Ev T
BCMA CAR T
BsAb-CAR T
97.2
2.8
62.4
37.6
5.02
97.6
2.35
61.3
38.7
4.38
97.2
2.75
72.3
27.7
5.61
97.8
2.18
97.1
2.9
32.4
Count
CD69-PE
hCD3-percp cy5.5

FIG. 7A

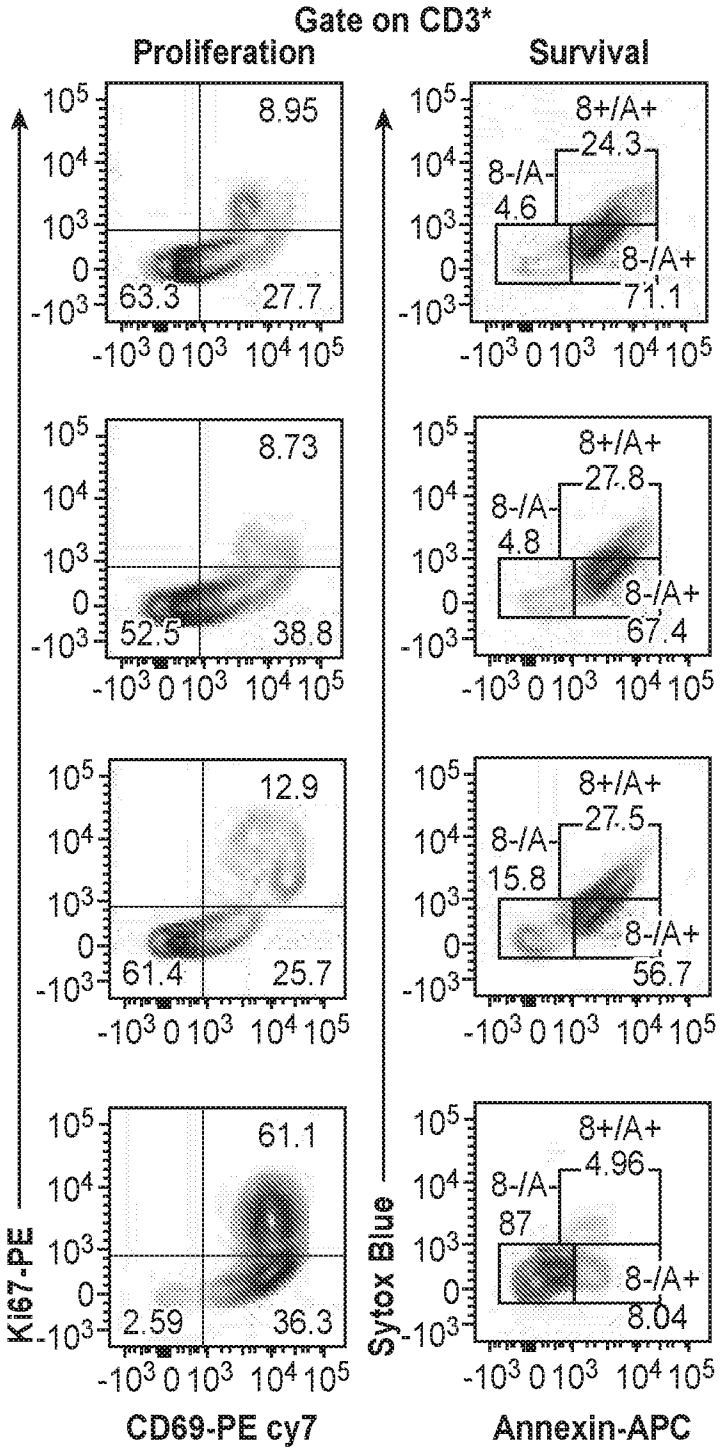

Gate on CD3*
Proliferation
Survival
8.95
63.3
27.7
8+/A+
24.3
8-/A-
4.6
8-/A+
71.1
8.73
52.5
38.8
8+/A+
27.8
8-/A-
4.8
8-/A+
67.4
12.9
61.4
25.7
8+/A+
27.5
8-/A-
15.8
8-/A+
56.7
61.1
2.59
36.3
8+/A+
4.96
8-/A-
87
8-/A+
8.04
Ki67-PE
Sytox Blue
CD69-PE cy7
Annexin-APC

FIG. 7A (Cont. 1)

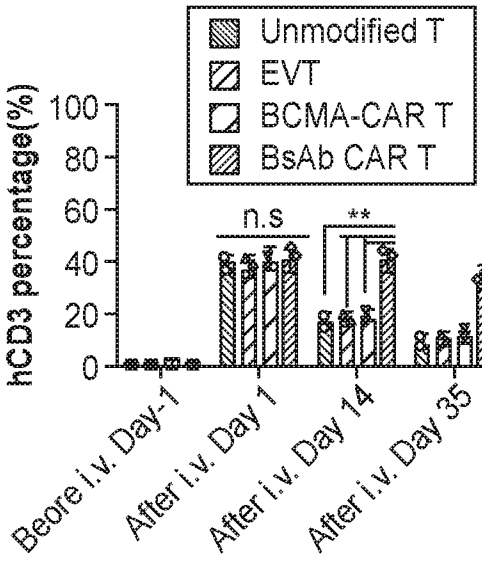



FIG. 7B

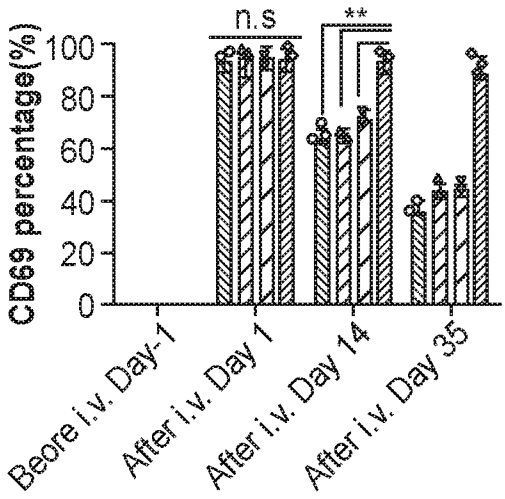



FIG. 7C

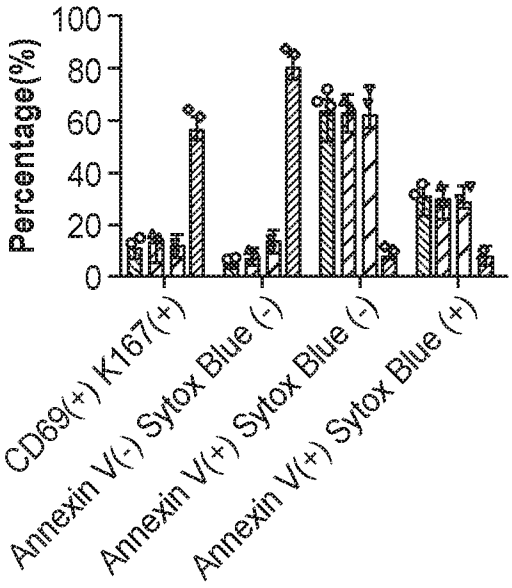



FIG. 7D

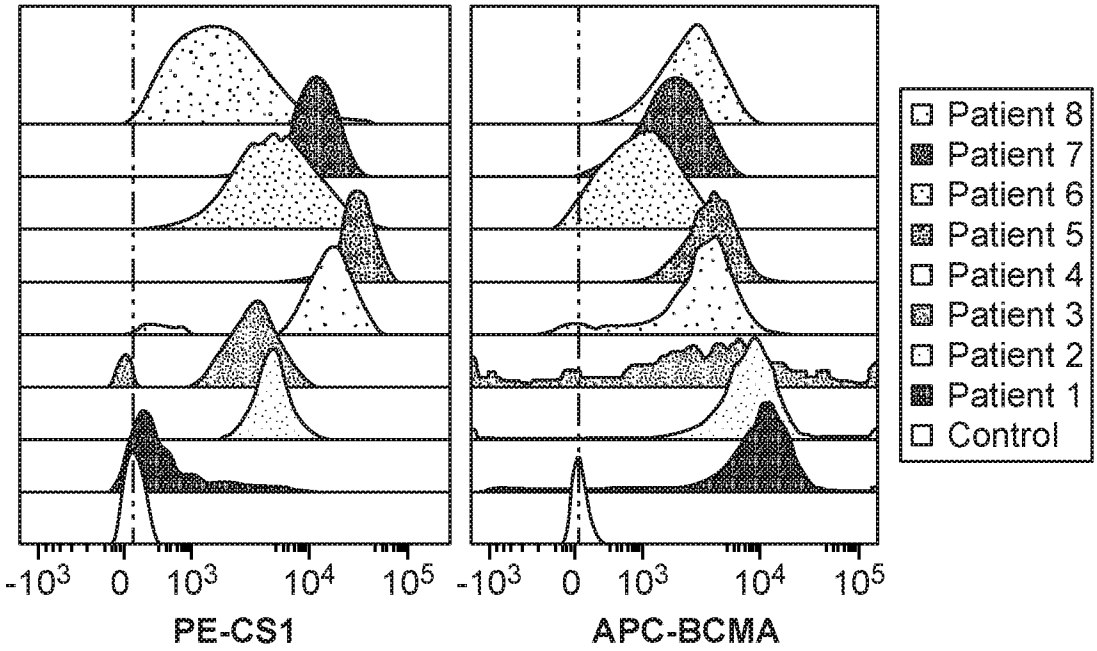

Patient 8
Patient 7
Patient 6
Patient 5
Patient 4
Patient 3
Patient 2
Patient 1
Control
-10^3 0 10^3 10^4 10^5
PE-CS1
-10^3 0 10^3 10^4 10^5
APC-BCMA

FIG. 8A

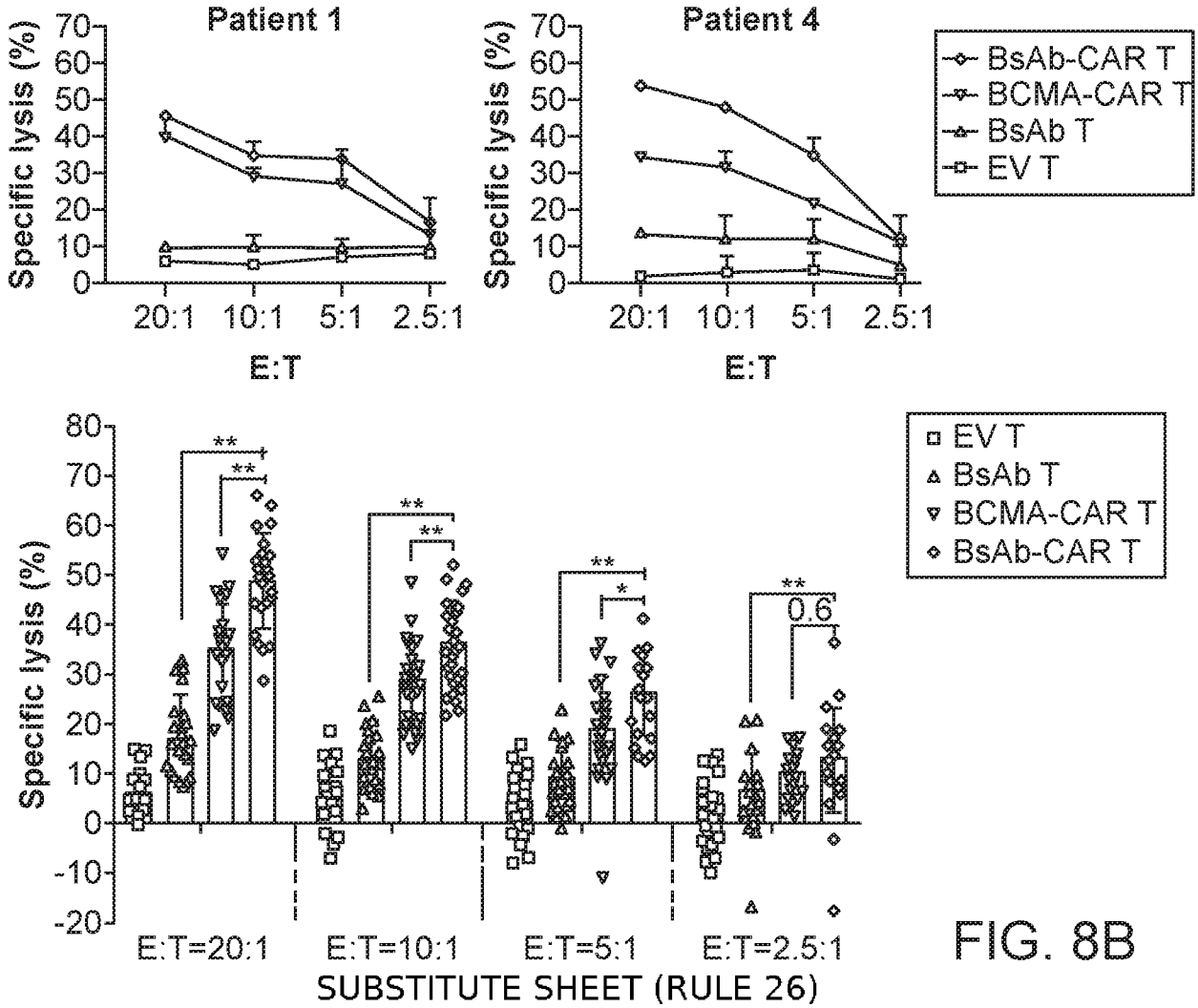

Patient 1
Specific lysis (%)
70
60
50
40
30
20
10
0
20:1 10:1 5:1 2.5:1
E:T
Patient 4
Specific lysis (%)
70
60
50
40
30
20
10
0
20:1 10:1 5:1 2.5:1
E:T
BsAb-CAR T
BCMA-CAR T
BsAb T
EV T
Specific lysis (%)
80
70
60
50
40
30
20
10
0
-10
-20
**
**
**
**
**
*
**
0.6
E:T=20:1
E:T=10:1
E:T=5:1
E:T=2.5:1
EV T
BsAb T
BCMA-CAR T
BsAb-CAR T

FIG. 8B

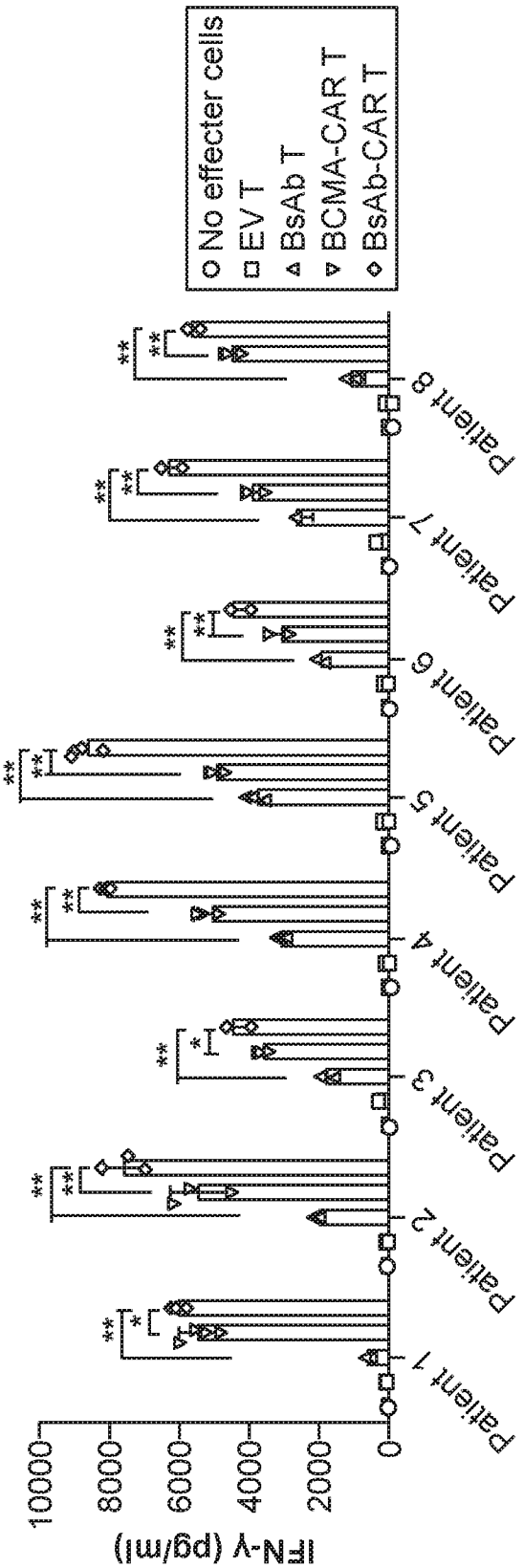

No effecter cells
EV T
BsAb T
BCMA-CAR T
BsAb-CAR T
IFN-γ (pg/ml)
10000
8000
6000
4000
2000
0
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
Patient 7
Patient 8

FIG. 8C

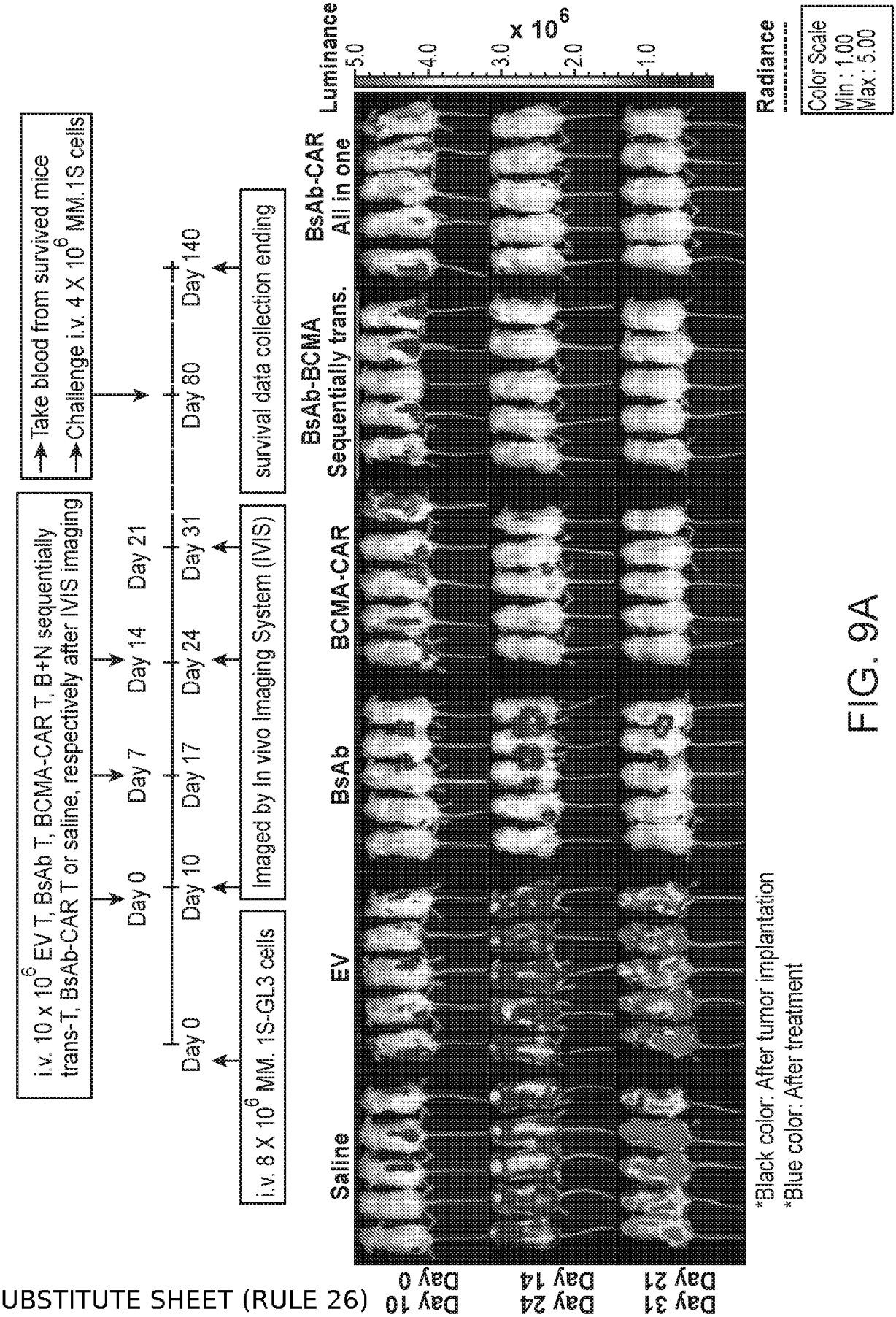

i.v. 10 x 10^6 EV T, BsAb T, BCMA-CAR T, B+N sequentially trans-T, BsAb-CAR T or saline, respectively after IVIS imaging
Take blood from survived mice
Challenge i.v. 4 X 10^6 MM.1S cells
Day 0
Day 7
Day 14
Day 21
Day 80
Day 140
Day 0
Day 10
Day 17
Day 24
Day 31
survival data collection ending
i.v. 8 X 10^6 MM. 1S-GL3 cells
Imaged by In vivo Imaging System (IVIS)
Saline
EV
BsAb
BCMA-CAR
BsAb-BCMA Sequentially trans.
BsAb-CAR All in one
Luminance
5.0
4.0
3.0
x 10^6
2.0
1.0
Radiance
Color Scale
Min : 1.00
Max : 5.00
Day 0
Day 10
Day 14
Day 24
Day 21
Day 31
*Black color: After tumor implantation
*Blue color: After treatment

FIG. 9A

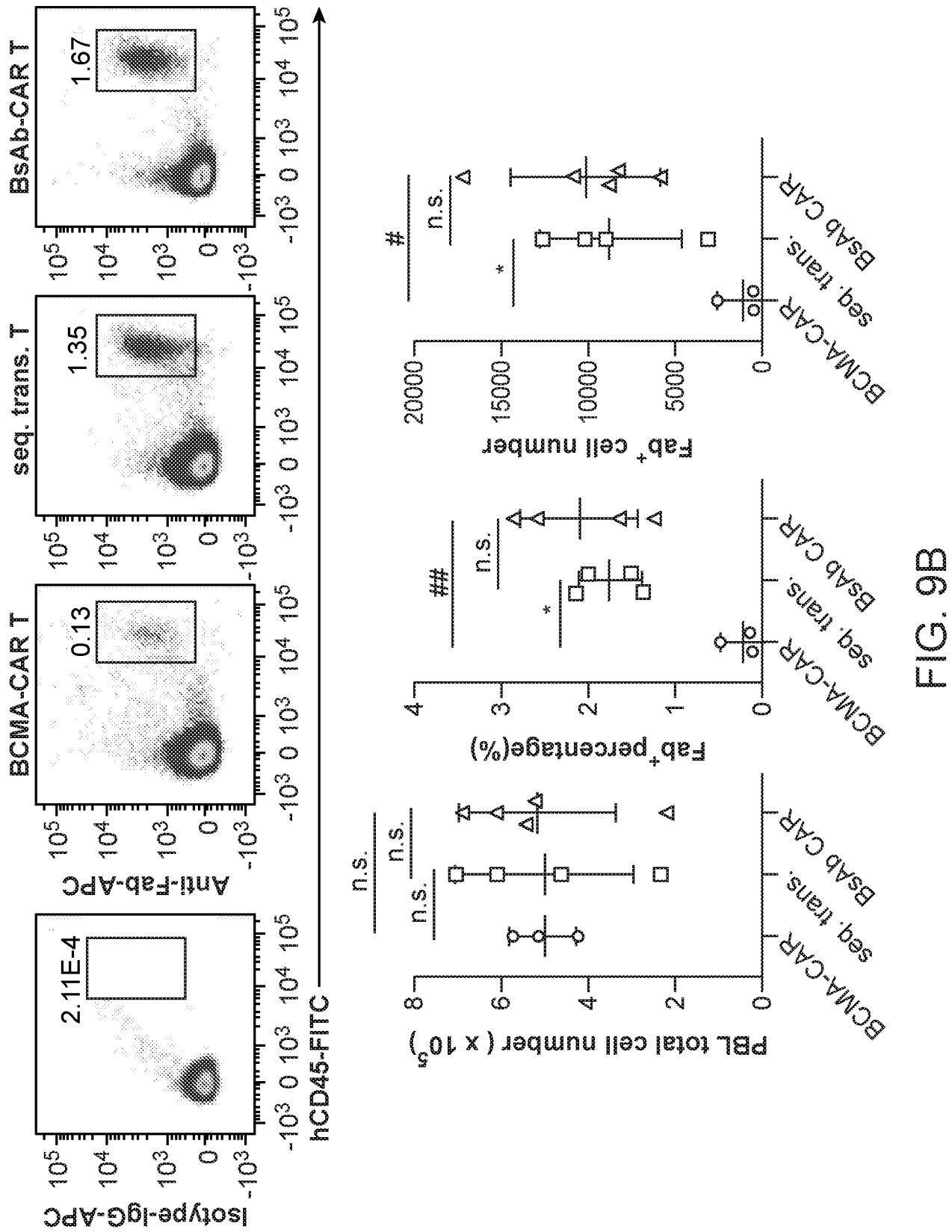

BCMA-CAR T
seq. trans. T
BsAb-CAR T
2.11E-4
0.13
1.35
1.67
Isotype-IgG-APC
Anti-Fab-APC
hCD45-FITC
PBL total cell number ( x 10^5)
Fab+percentage(%)
Fab+ cell number
n.s.
##
#
*
BCMA-CAR
seq. trans. BsAb CAR

FIG. 9B

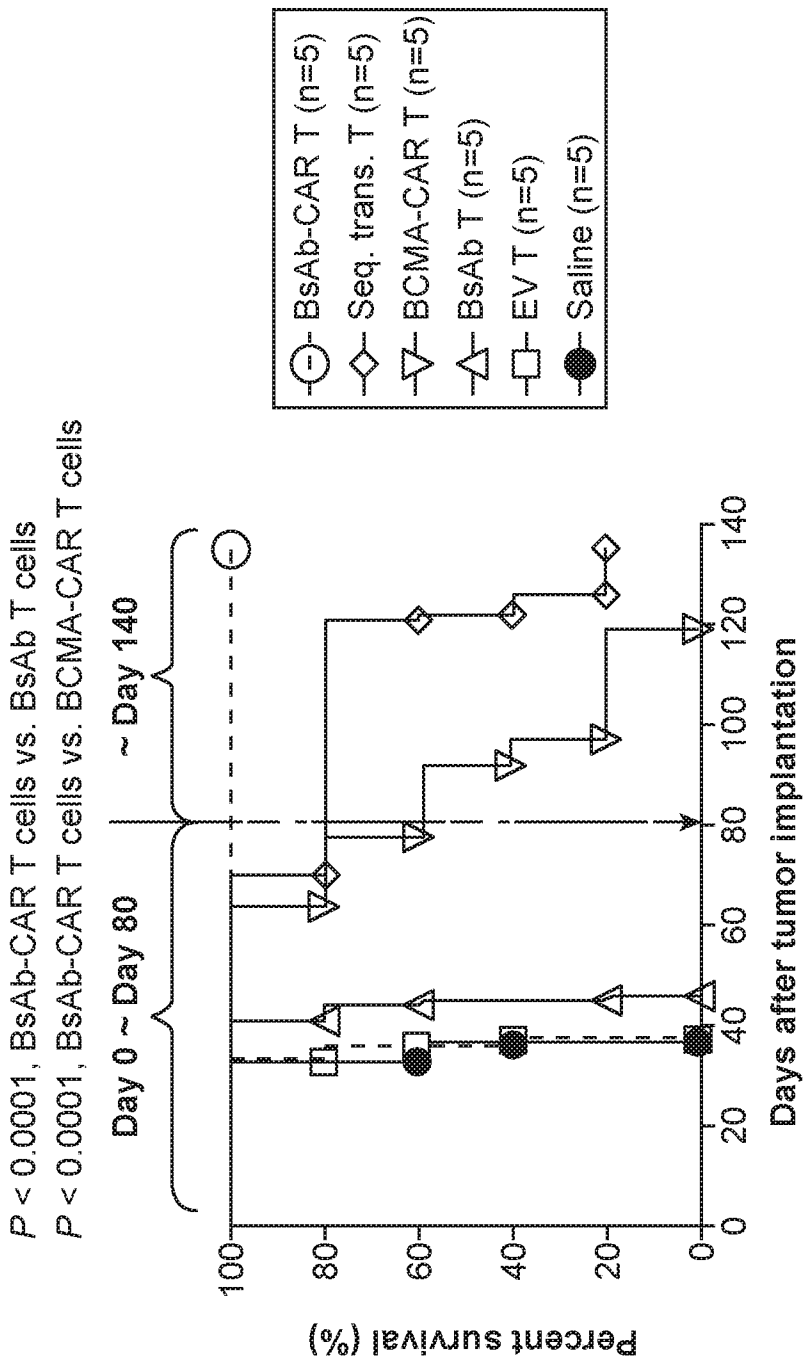

BsAb-CAR T (n=5)
Seq. trans. T (n=5)
BCMA-CAR T (n=5)
BsAb T (n=5)
EV T (n=5)
Saline (n=5)
P < 0.0001, BsAb-CAR T cells vs. BsAb T cells
P < 0.0001, BsAb-CAR T cells vs. BCMA-CAR T cells
Day 0 ~ Day 80
~ Day 140
Percent survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Days after tumor implantation

FIG. 9C

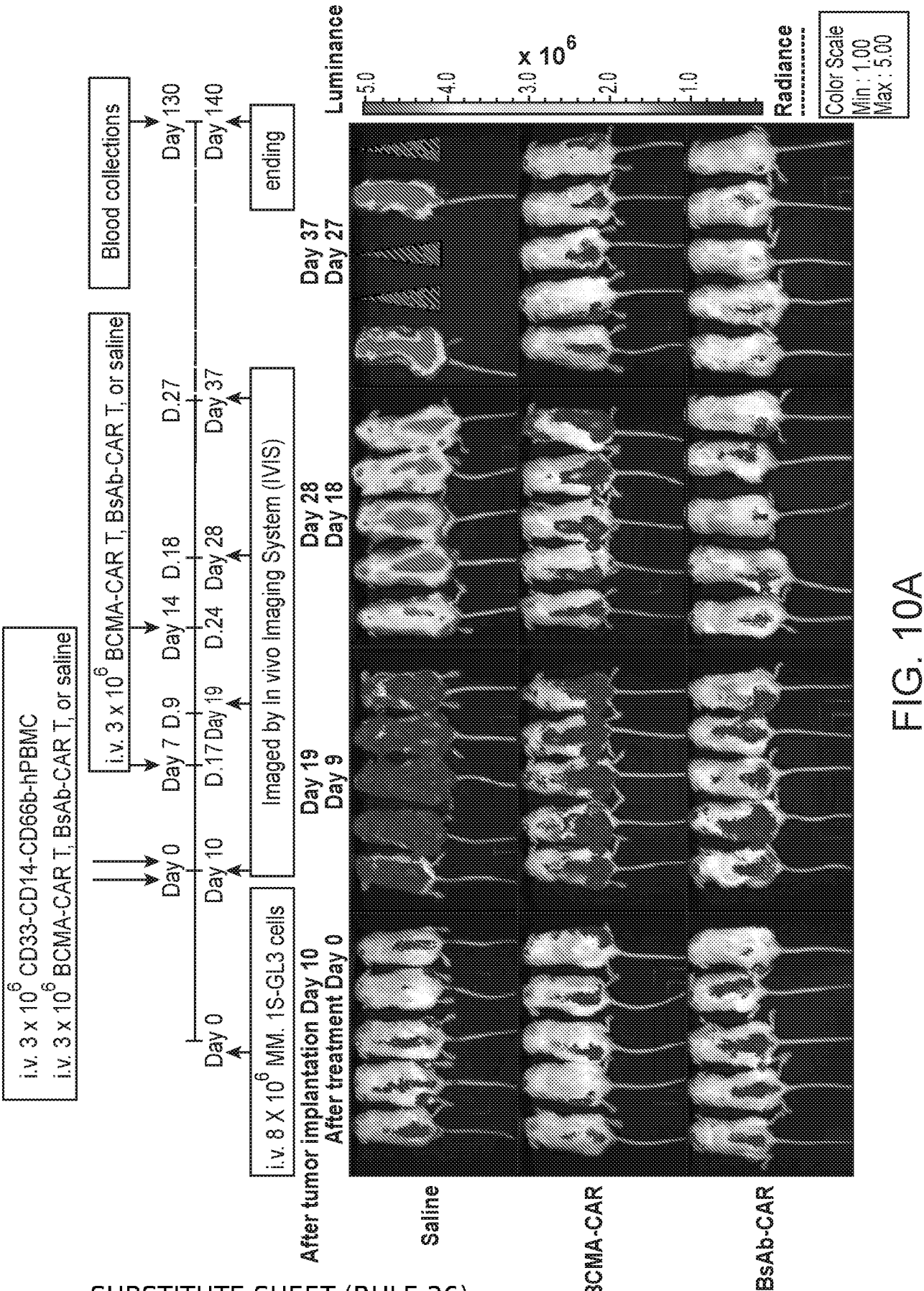

i.v. 3 x 10^6 CD33-CD14-CD66b-hPBMC
i.v. 3 x 10^6 BCMA-CAR T, BsAb-CAR T, or saline
i.v. 3 x 10^6 BCMA-CAR T, BsAb-CAR T, or saline
Blood collections
ending
i.v. 8 X 10^6 MM. 1S-GL3 cells
Imaged by in vivo Imaging System (IVIS)
Day 0
Day 10
Day 7
D.9
D.17
Day 19
Day 14
D.18
D.24
Day 28
D.27
Day 37
Day 130
Day 140
After tumor implantation Day 10
After treatment Day 0
Day 19
Day 9
Day 28
Day 18
Day 37
Day 27
Luminance
x 10^6
5.0
4.0
3.0
2.0
1.0
Radiance
Color Scale
Min : 1.00
Max : 5.00
Saline
BCMA-CAR
BsAb-CAR

FIG. 10A

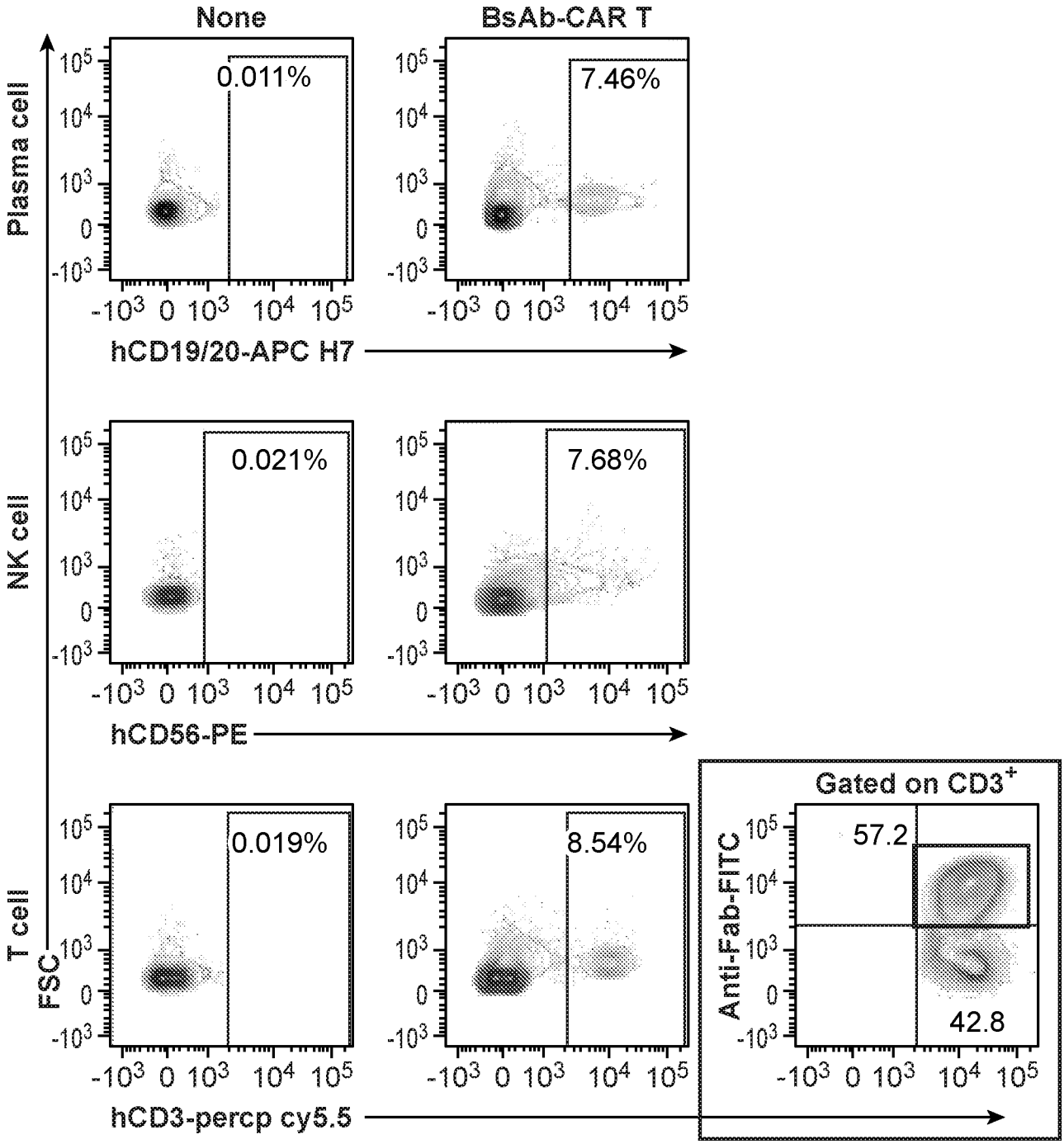

None
BsAb-CAR T
Plasma cell
0.011%
7.46%
hCD19/20-APC H7
NK cell
0.021%
7.68%
hCD56-PE
T cell
FSC
0.019%
8.54%
hCD3-percp cy5.5
Gated on CD3+
57.2
42.8
Anti-Fab-FITC

FIG. 10B

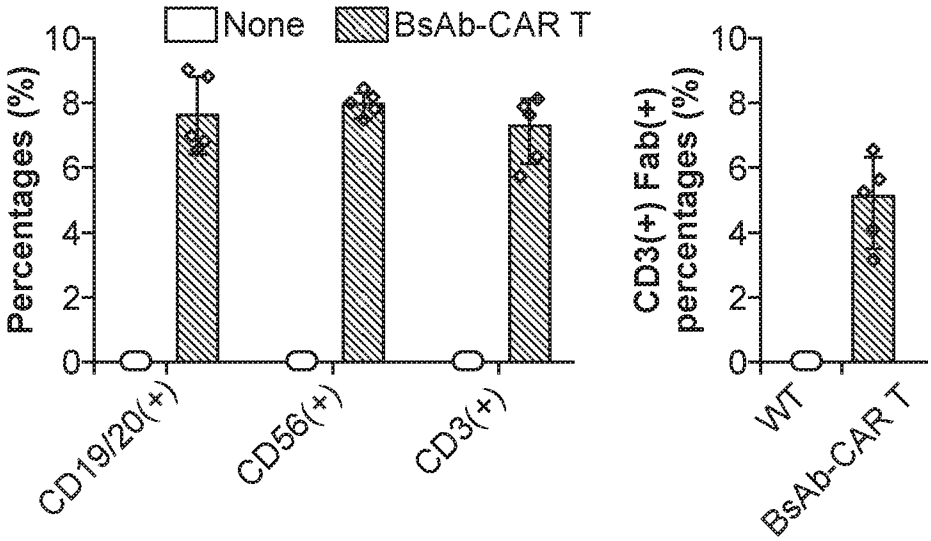



FIG. 10C

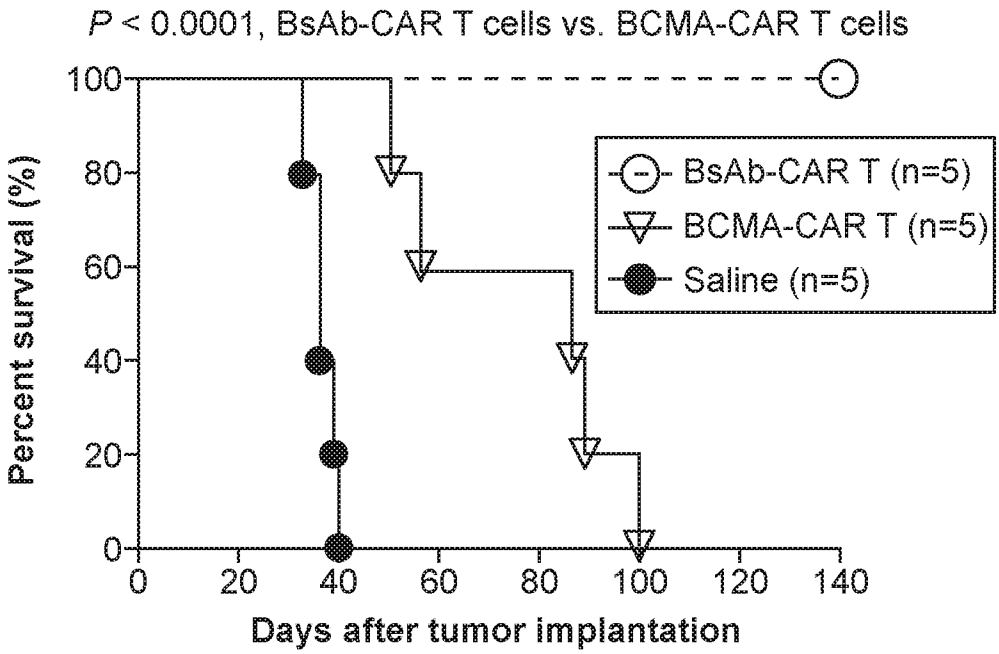
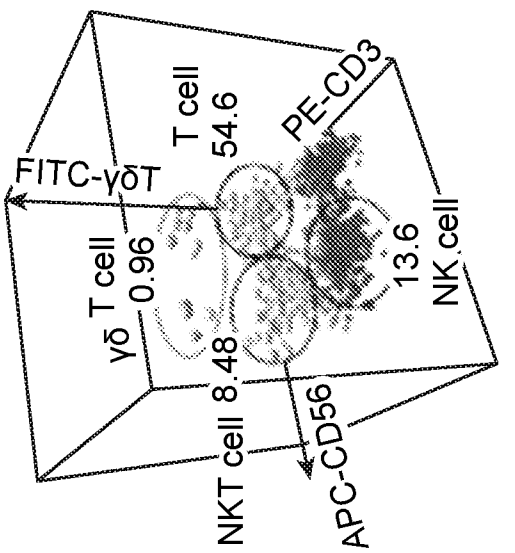
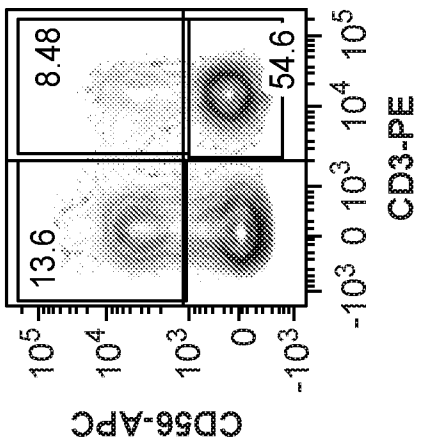



FIG. 10D



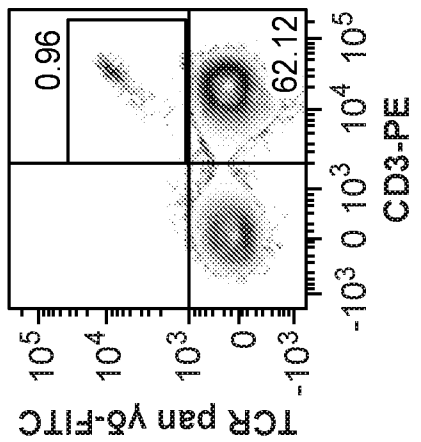




FIG. 11A

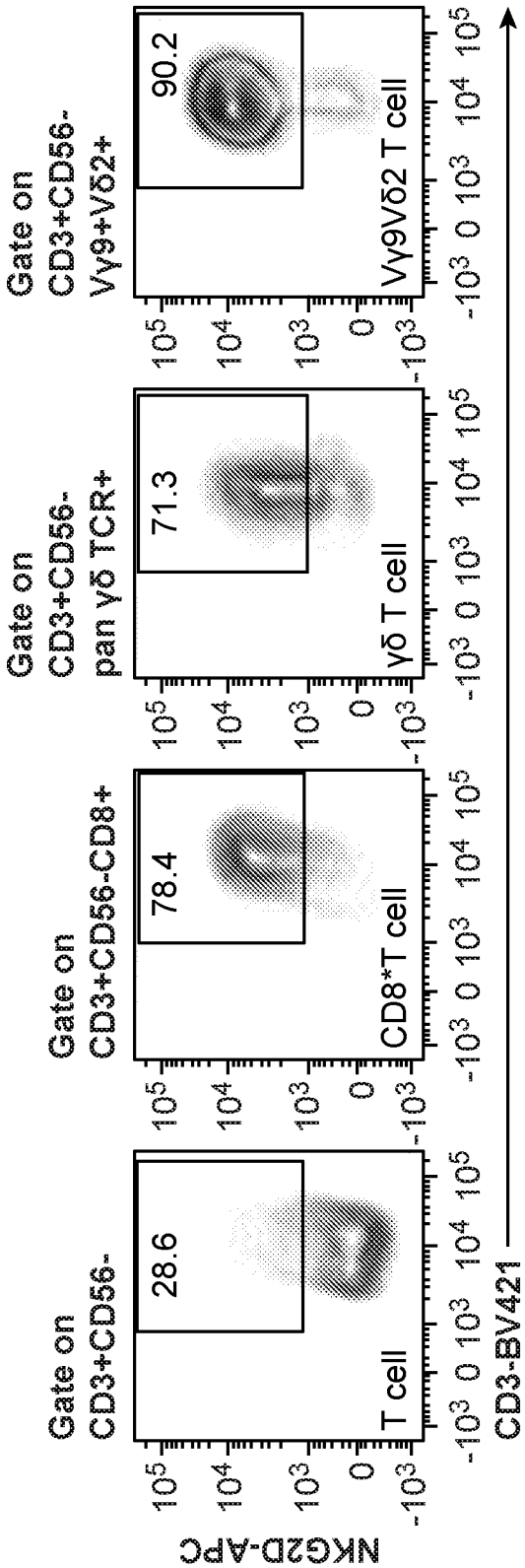



FIG. 11B

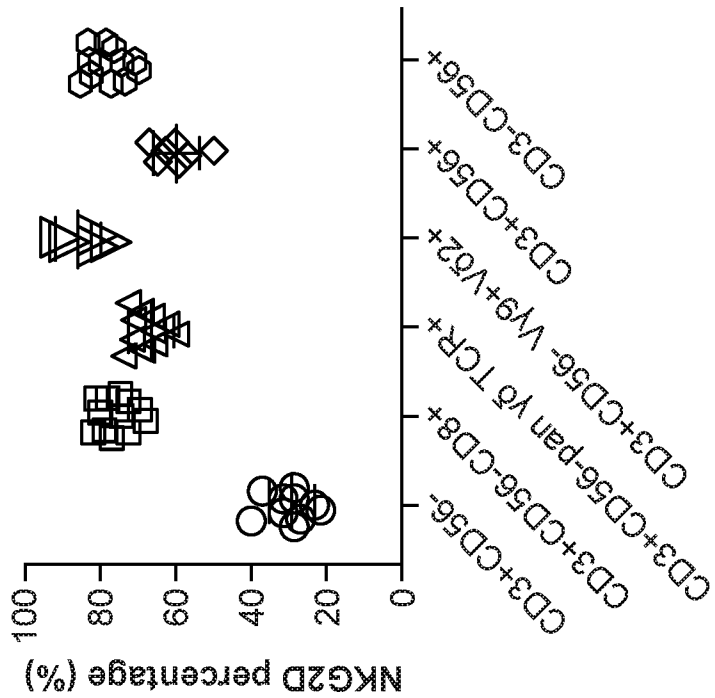



FIG. 11C

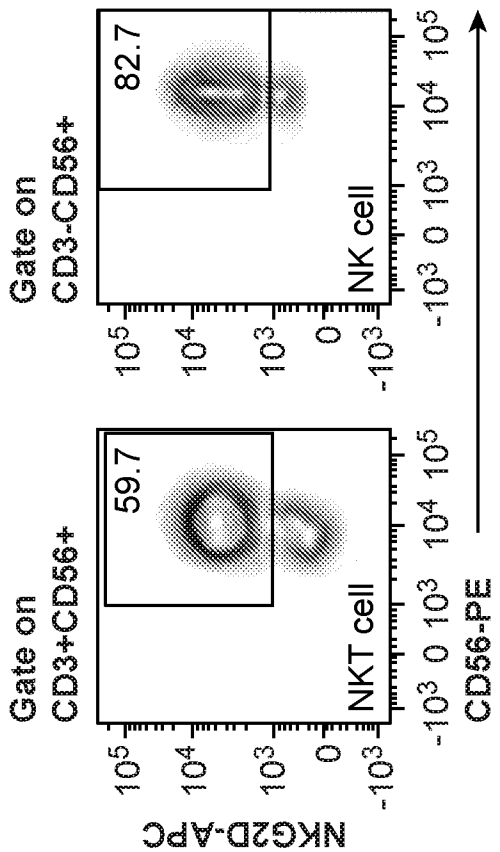



FIG. 11B (Cont.)

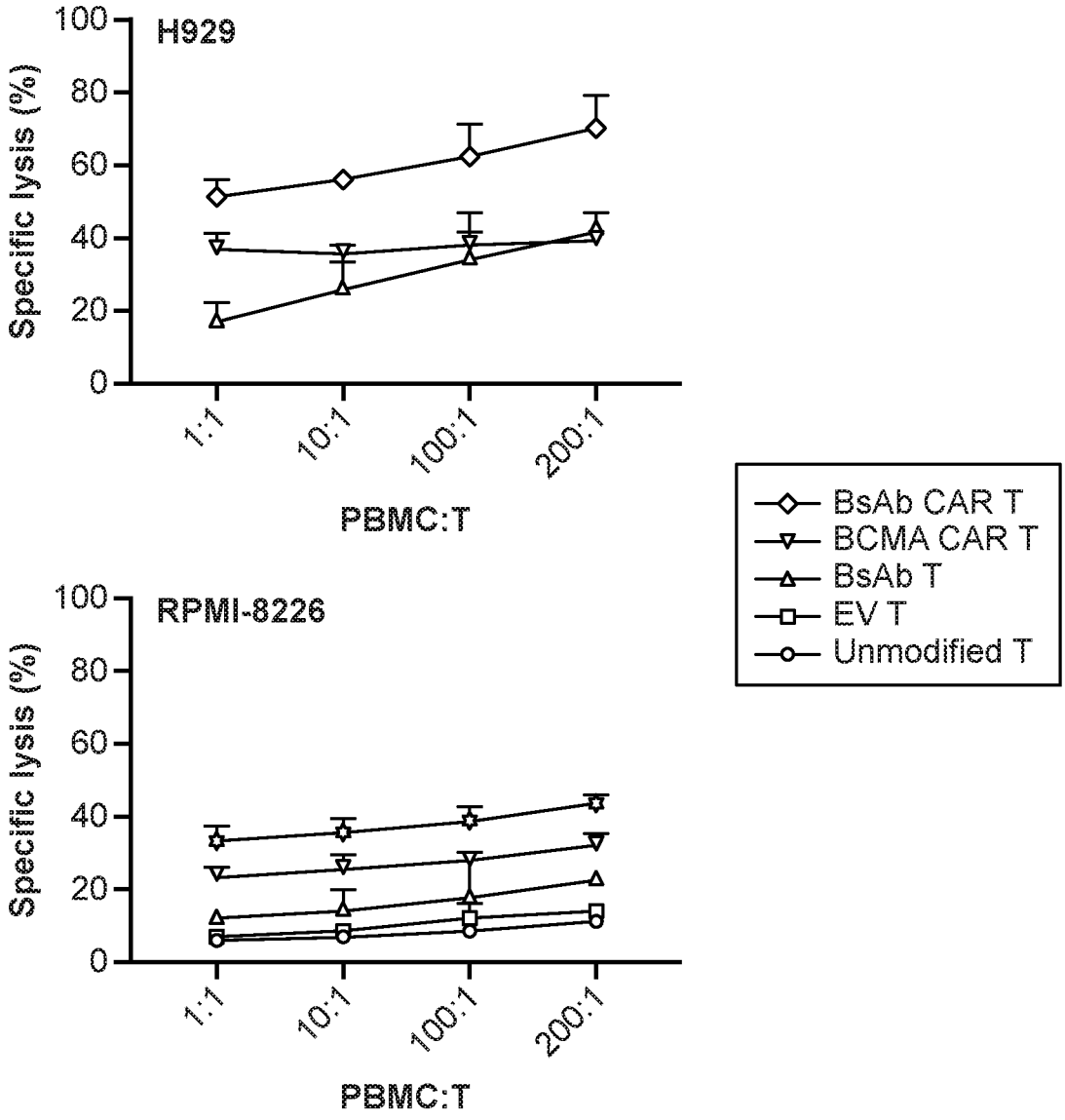

H929
Specific lysis (%)
100
80
60
40
20
0
1:1
10:1
100:1
200:1
PBMC:T
RPMI-8226
Specific lysis (%)
100
80
60
40
20
0
1:1
10:1
100:1
200:1
PBMC:T
BsAb CAR T
BCMA CAR T
BsAb T
EV T
Unmodified T

FIG. 12A

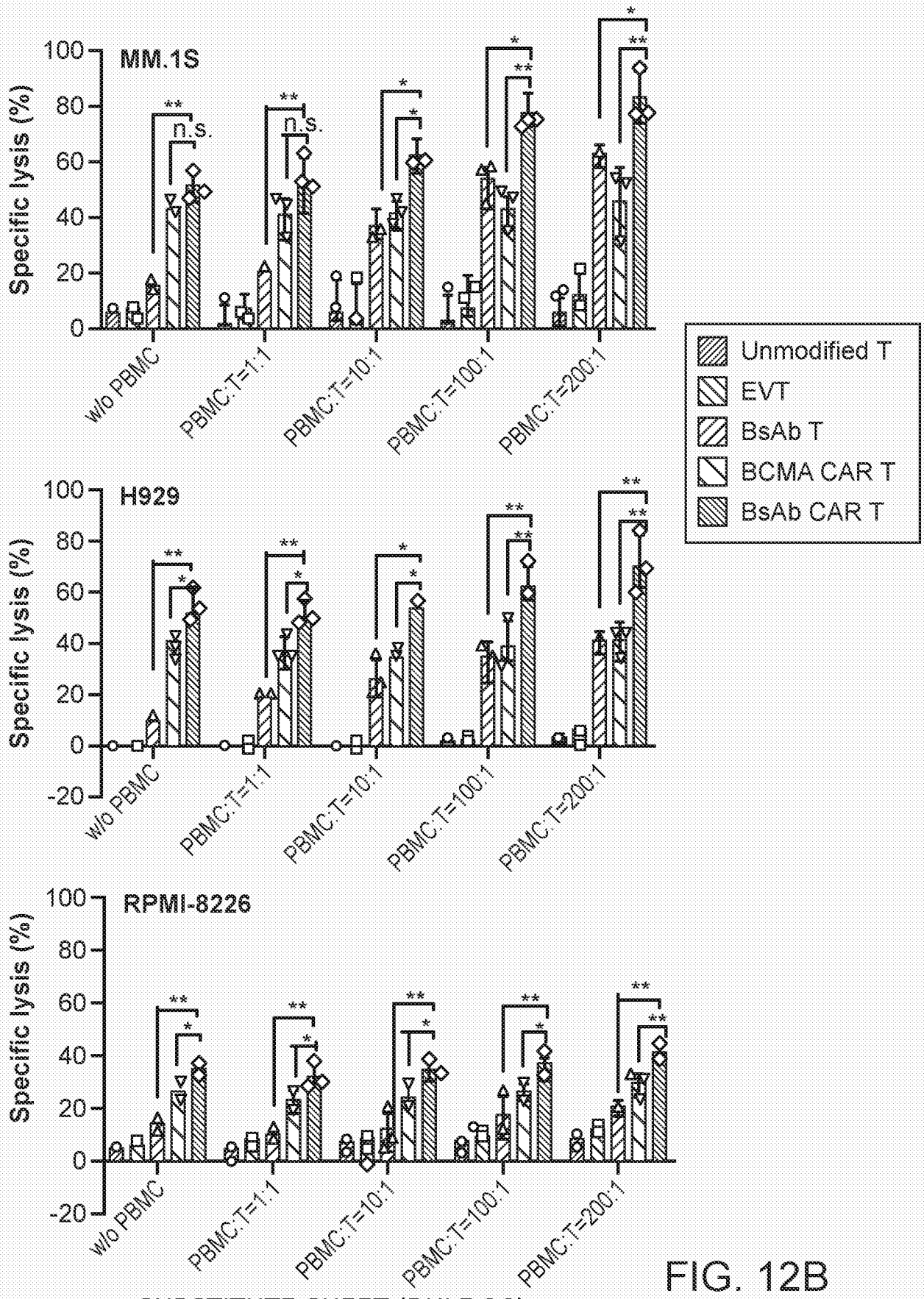

MM.1S
H929
RPMI-8226
Specific lysis (%)
100
80
60
40
20
0
-20
n.s.
w/o PBMC
PBMC:T=1:1
PBMC:T=10:1
PBMC:T=100:1
PBMC:T=200:1
Unmodified T
EVT
BsAb T
BCMA CAR T
BsAb CAR T

FIG. 12B

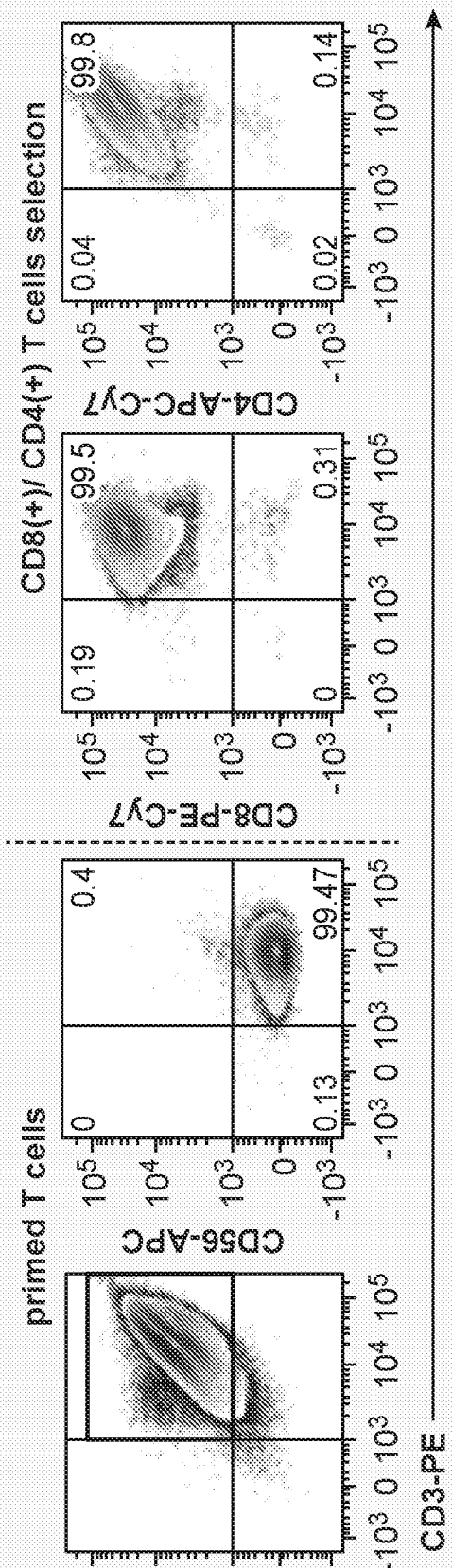



FIG. 13A

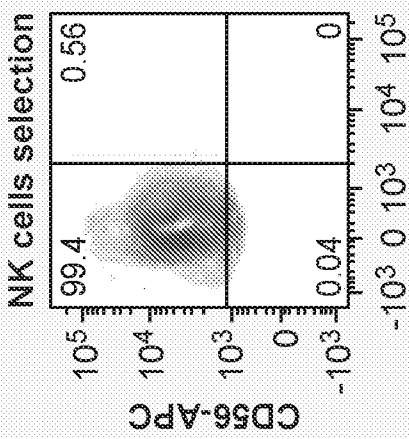



FIG. 13B

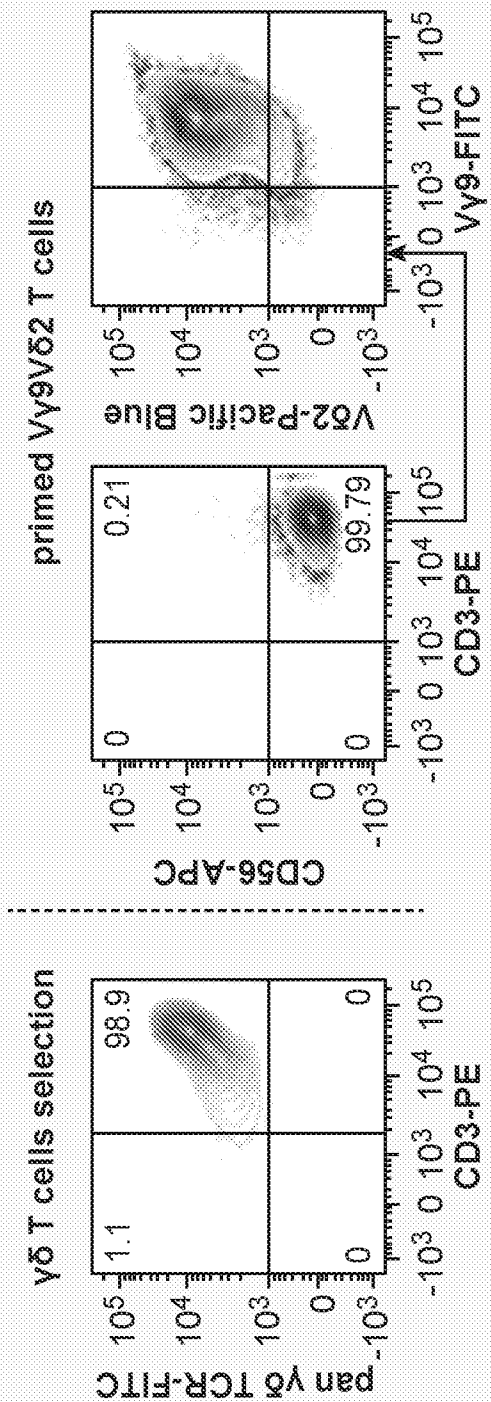



FIG. 13C

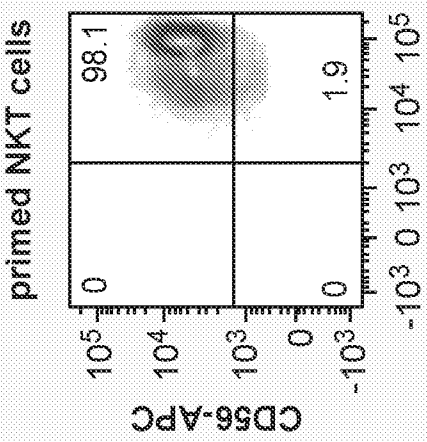



FIG. 13D

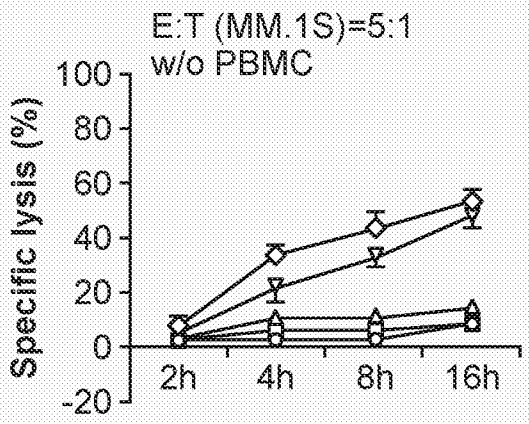



FIG. 14A

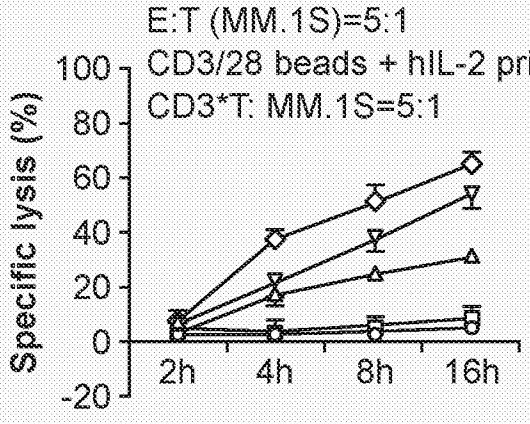



FIG. 14B

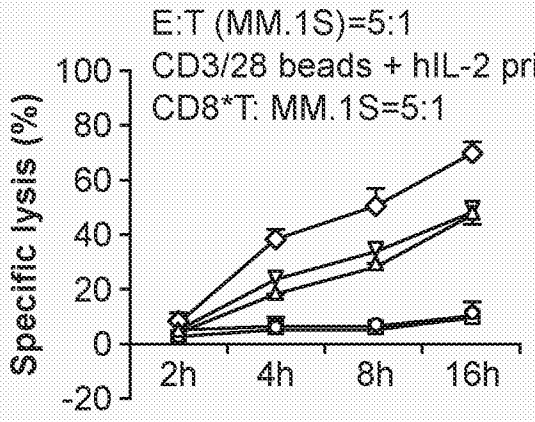



FIG. 14C

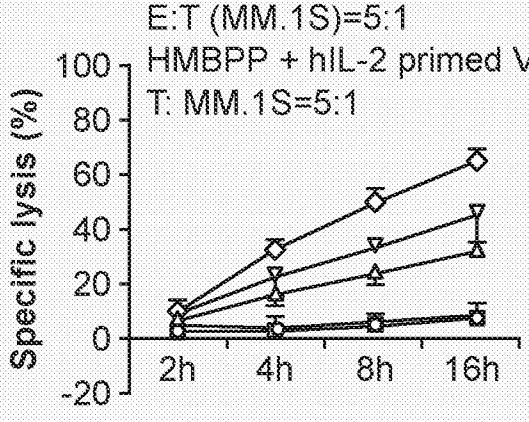



FIG. 14D

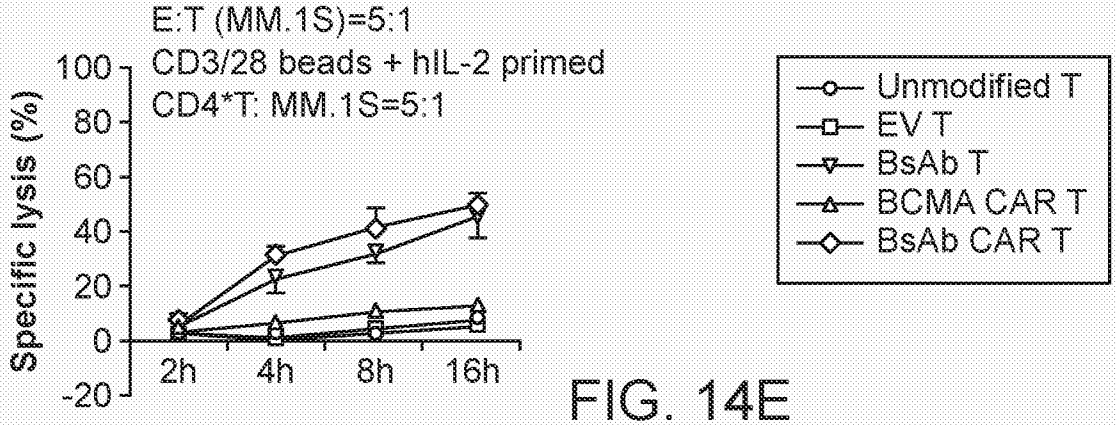



FIG. 14E

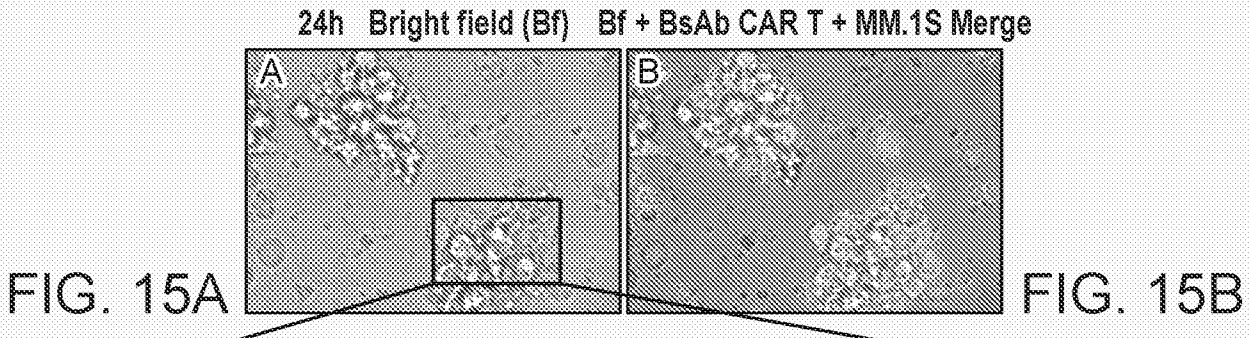



FIG. 15A FIG. 15B

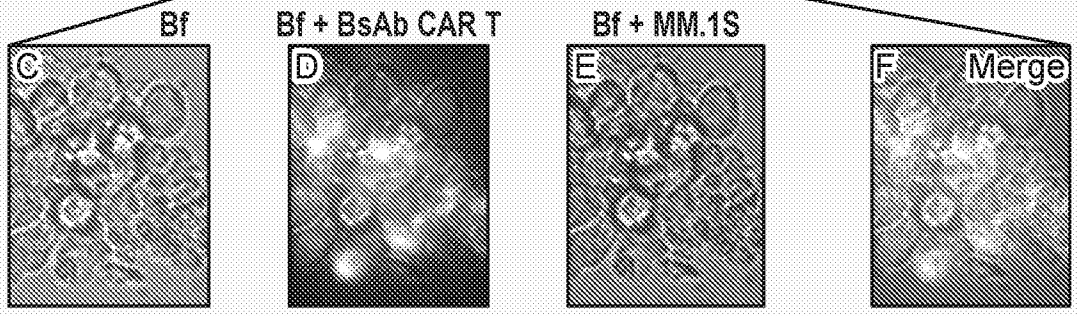



FIG. 15C FIG. 15D FIG. 15E FIG. 15F

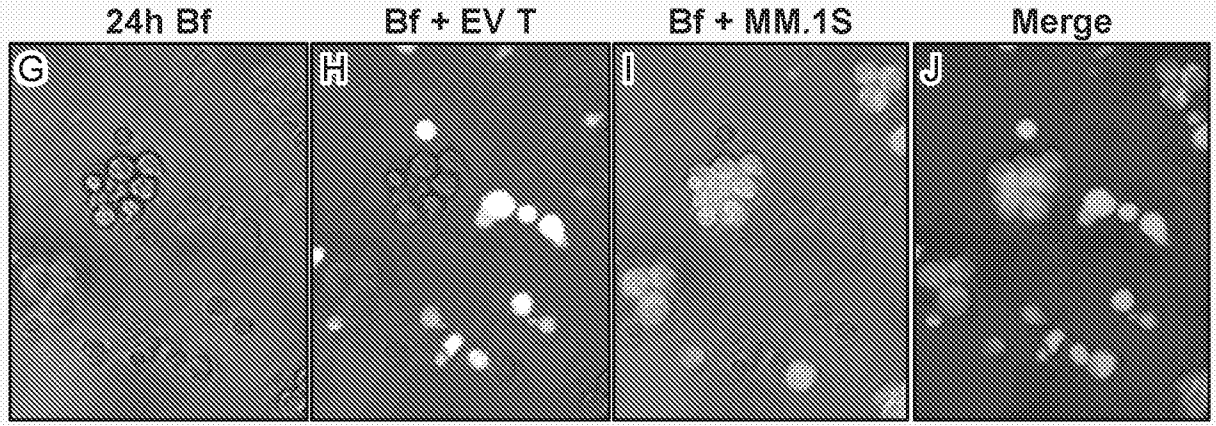



FIG. 15G FIG. 15H FIG. 15I FIG. 15J

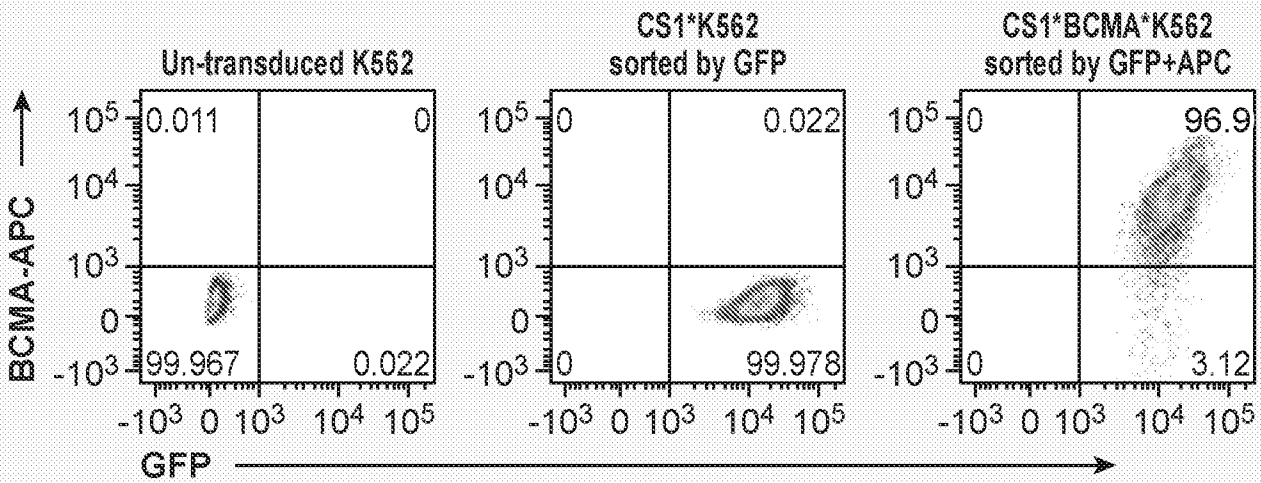



 FIG. 16

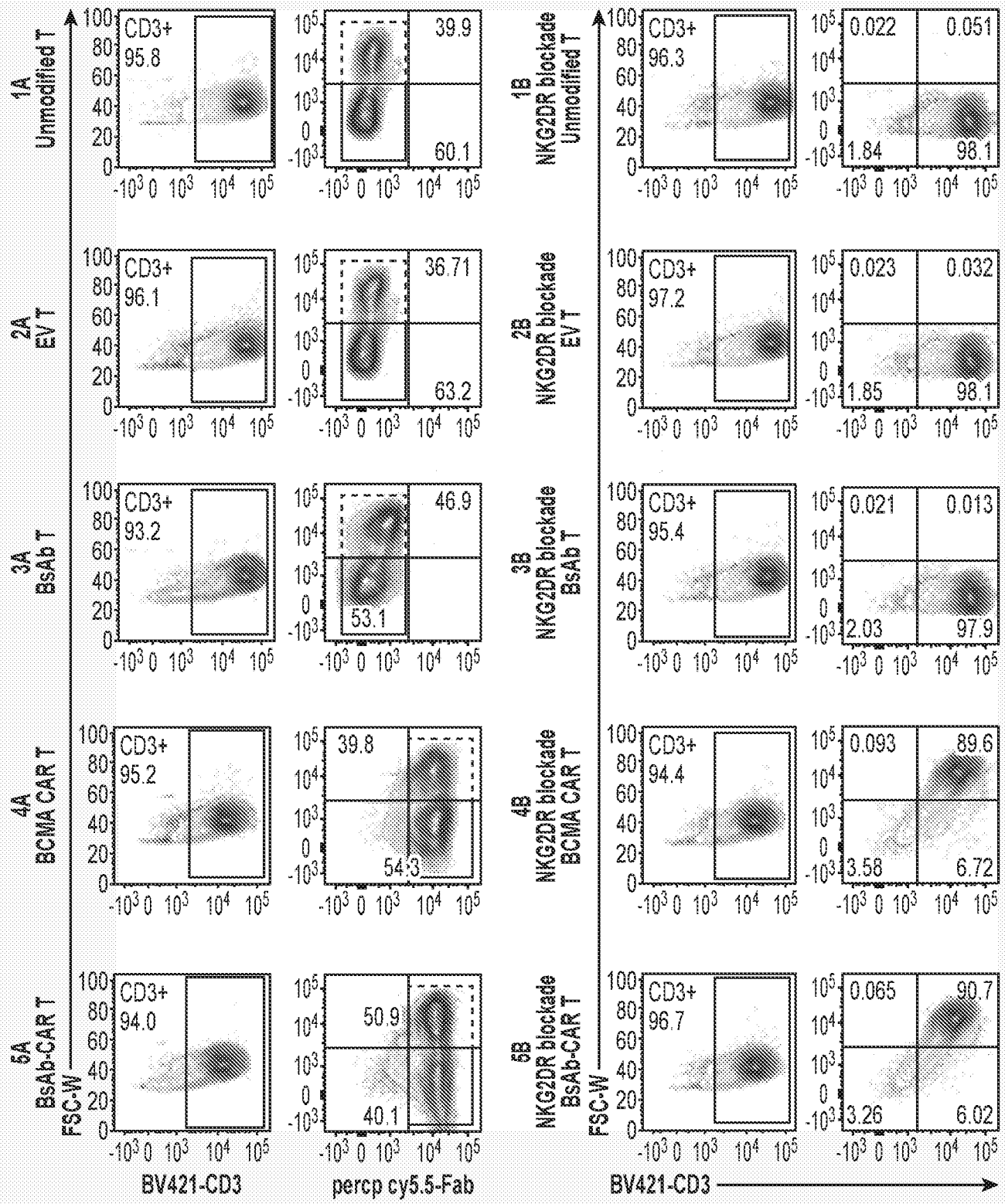



FIG. 17A FIG. 17B

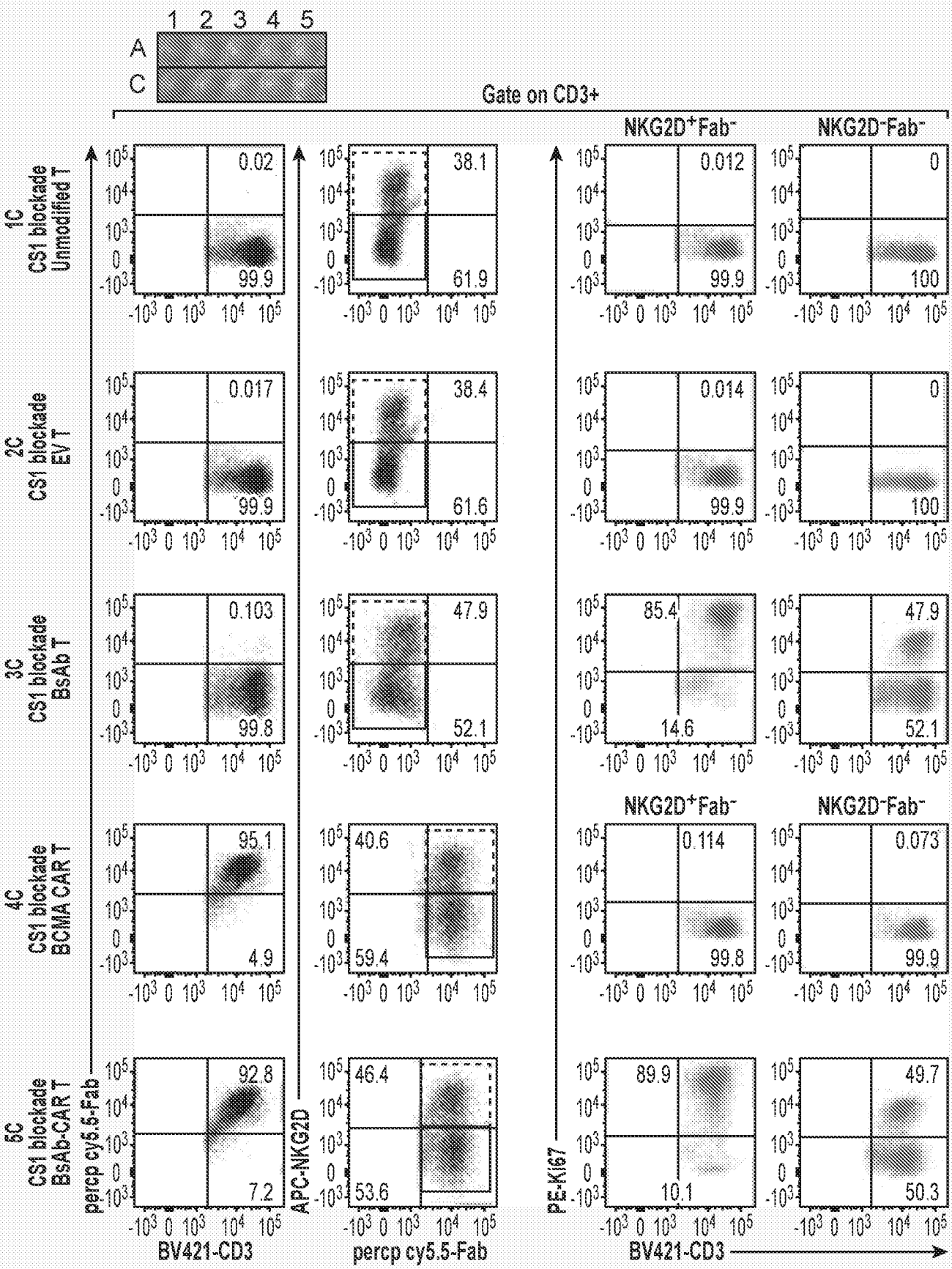

1 2 3 4 5
A
C
Gate on CD3+
NKG2D+Fab-
NKG2D-Fab-
1C CS1 blockade Unmodified T
2C CS1 blockade EV T
3C CS1 blockade BsAb T
4C CS1 blockade BCMA CAR T
5C CS1 blockade BsAb-CAR T
0.02
99.9
38.1
61.9
0.012
99.9
0
100
0.017
99.9
38.4
61.6
0.014
99.9
0
100
0.103
99.8
47.9
52.1
85.4
14.6
47.9
52.1
95.1
4.9
40.6
59.4
0.114
99.8
0.073
99.9
92.8
7.2
46.4
53.6
89.9
10.1
49.7
50.3
percp cy5.5-Fab
BV421-CD3
APC-NKG2D
percp cy5.5-Fab
PE-Ki67
BV421-CD3

FIG. 17C

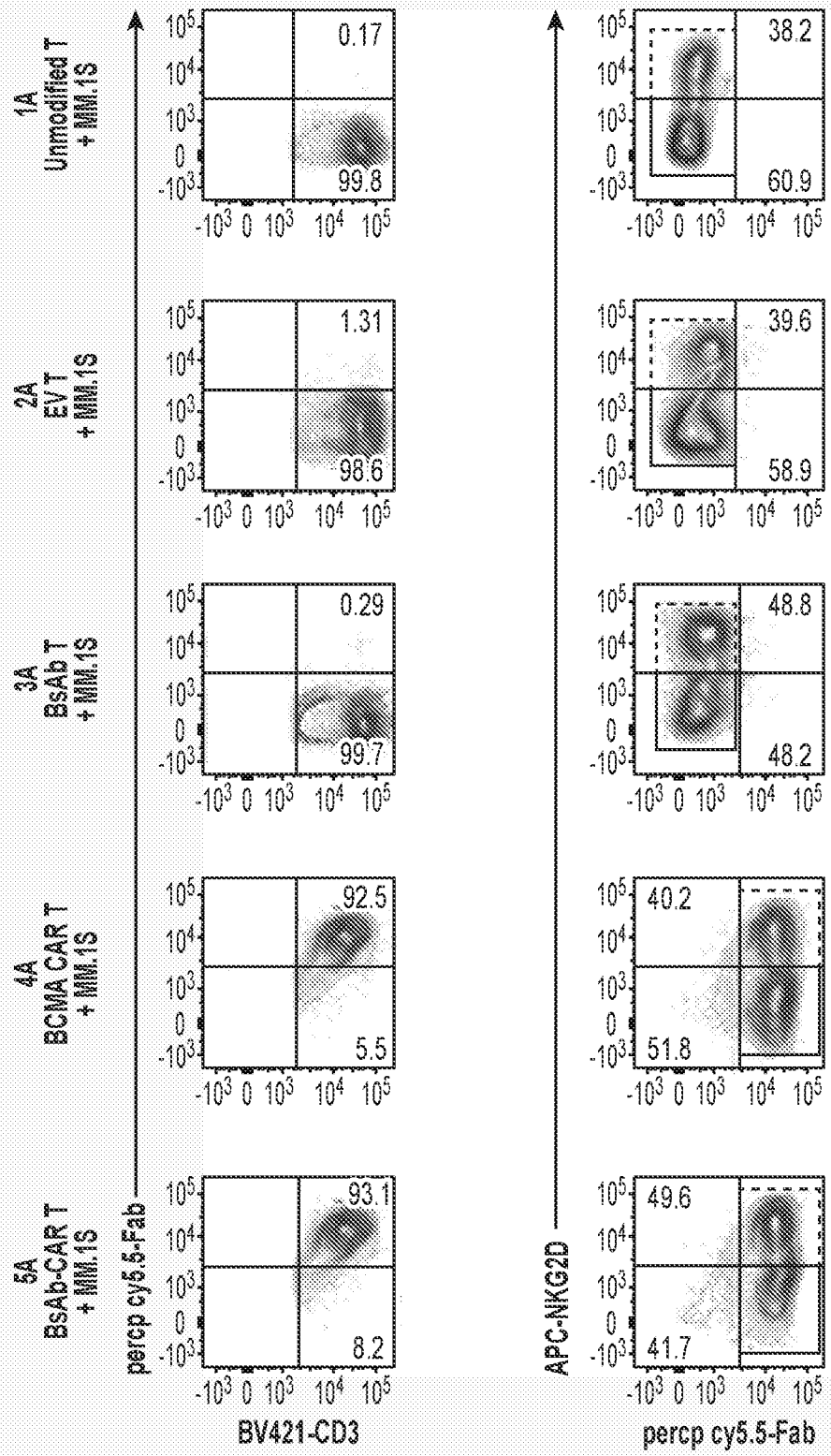

1A
Unmodified T
+ MM.1S
2A
EV T
+ MM.1S
3A
BsAb T
+ MM.1S
4A
BCMA CAR T
+ MM.1S
5A
BsAb-CAR T
+ MM.1S
0.17
99.8
1.31
98.6
0.29
99.7
92.5
5.5
93.1
8.2
38.2
60.9
39.6
58.9
48.8
48.2
40.2
51.8
49.6
41.7
percp cy5.5-Fab
BV421-CD3
APC-NKG2D
percp cy5.5-Fab

FIG. 17D

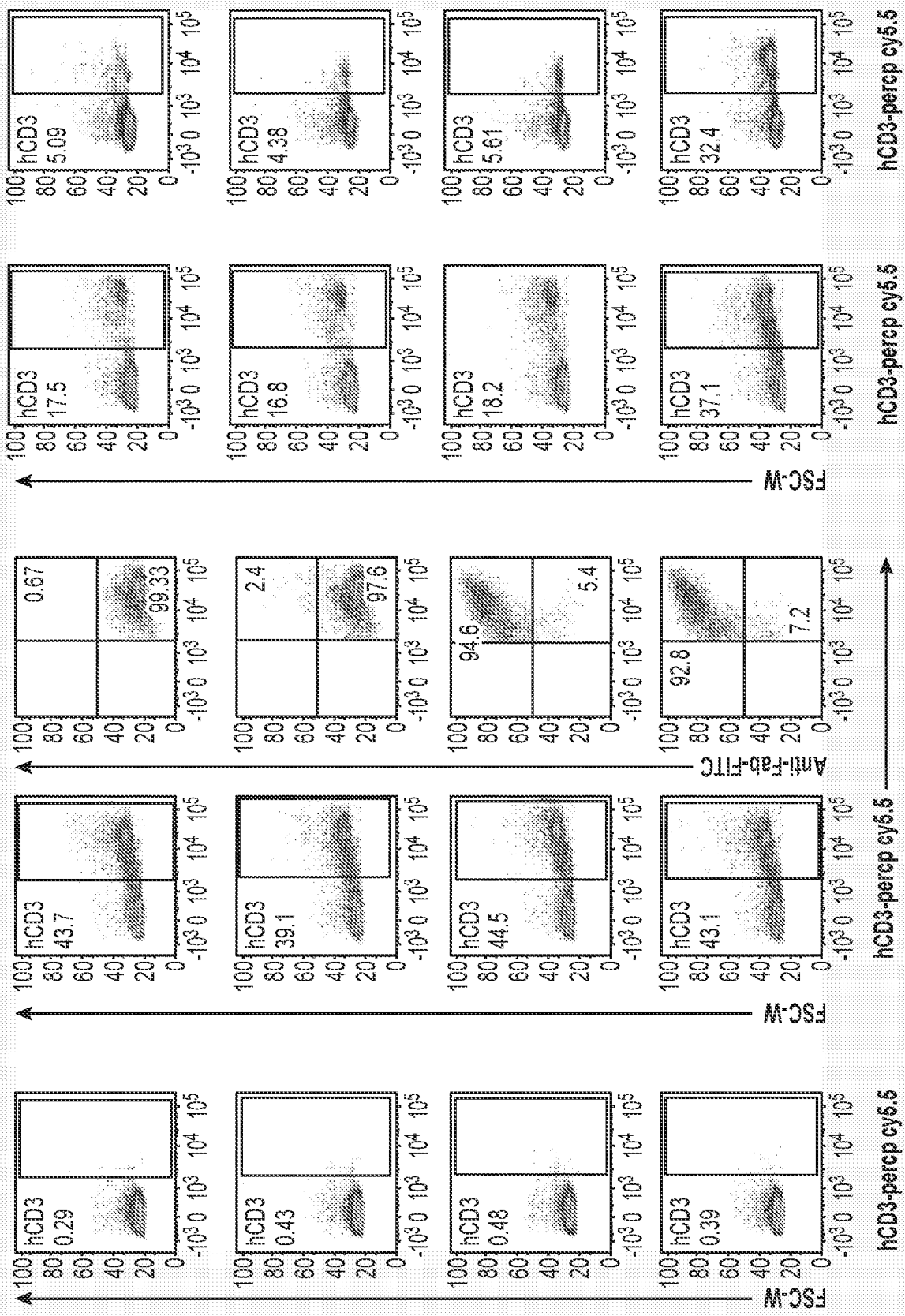

Before i.v. Day-1
After i.v. Day 1
After i.v. Day 14
After i.v. Day 35
Unmodified T
EV T
BCMA CAR T
BsAb-CAR T
FSC-W
hCD3-percp cy5.5
Anti-Fab-FITC
hCD3
0.29
0.43
0.48
0.39
43.7
39.1
44.5
43.1
0.67
99.33
2.4
97.6
94.6
5.4
92.8
7.2
17.5
16.8
18.2
37.1
5.09
4.38
5.61
32.4

FIG. 18

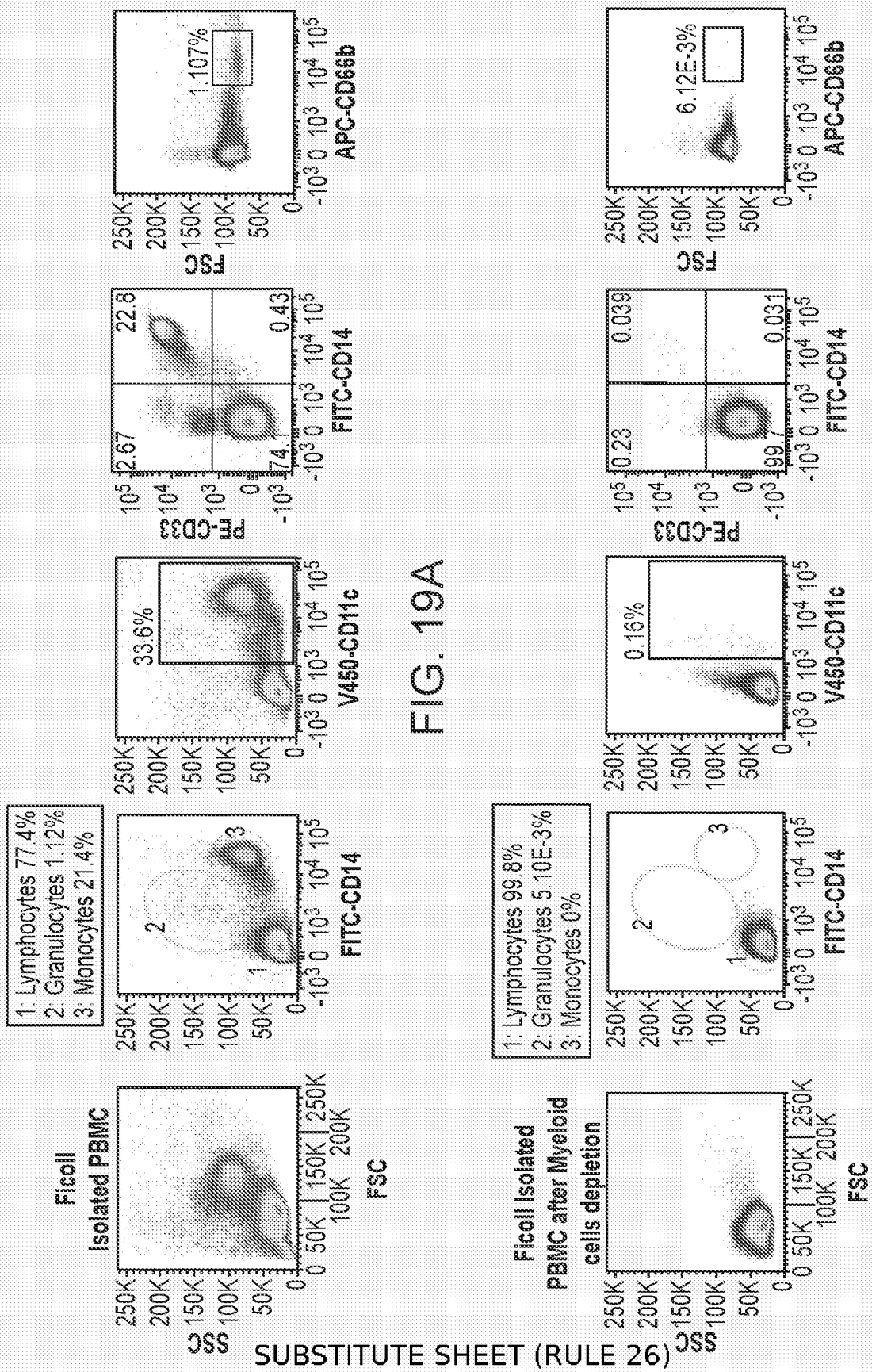



FIG. 19A

FIG. 19B

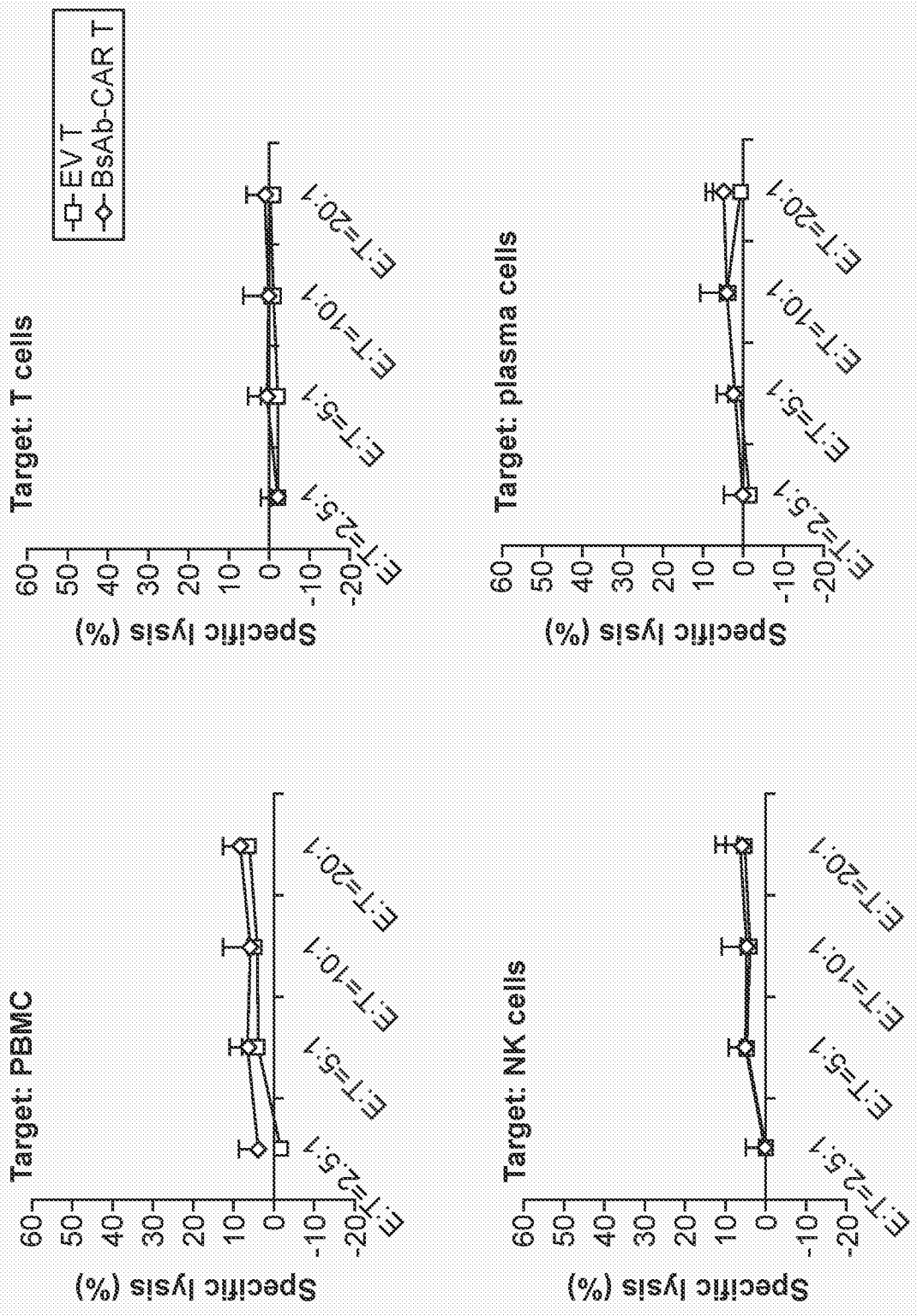

EVT
BsAb-CAR T
Target: T cells
Target: plasma cells
Target: PBMC
Target: NK cells
Specific lysis (%)
60
50
40
30
20
10
0
-10
-20
E:T=2.5:1
E:T=5:1
E:T=10:1
E:T=20:1

FIG. 20

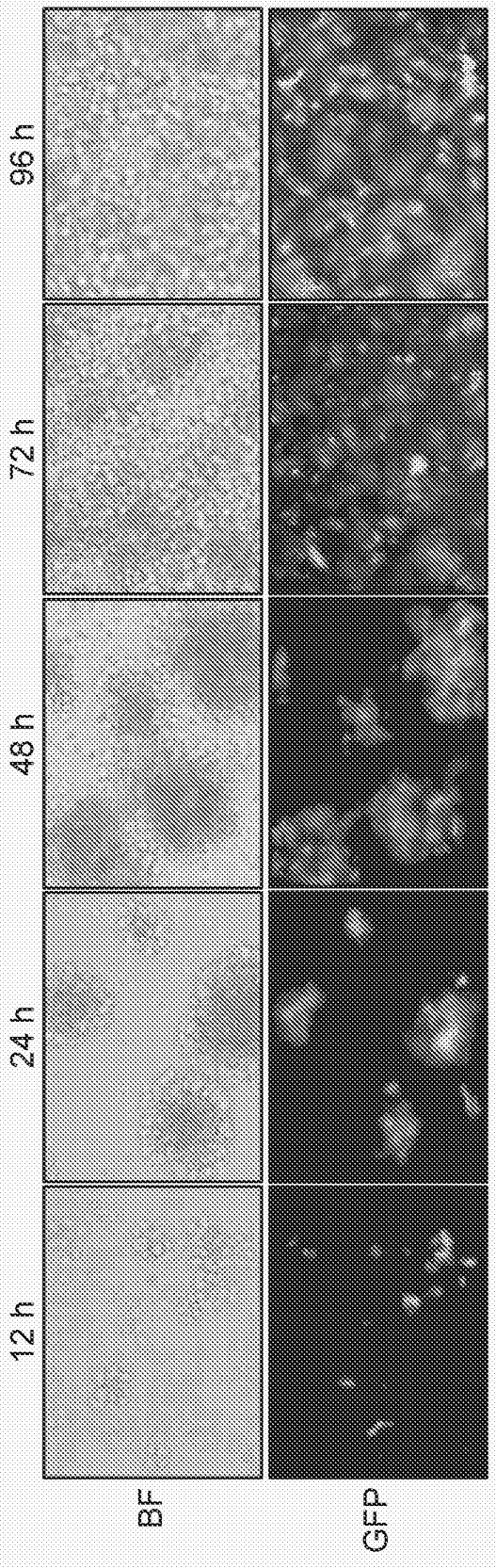



FIG. 21A

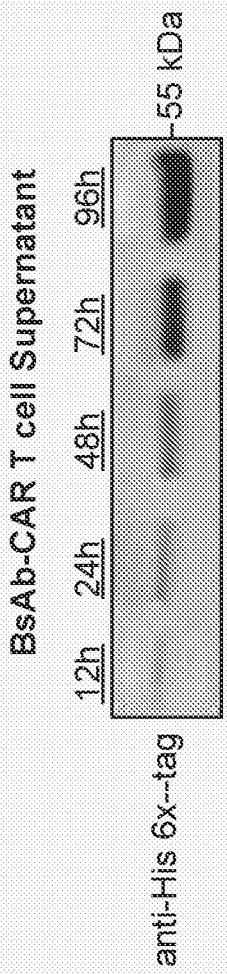



FIG. 21B

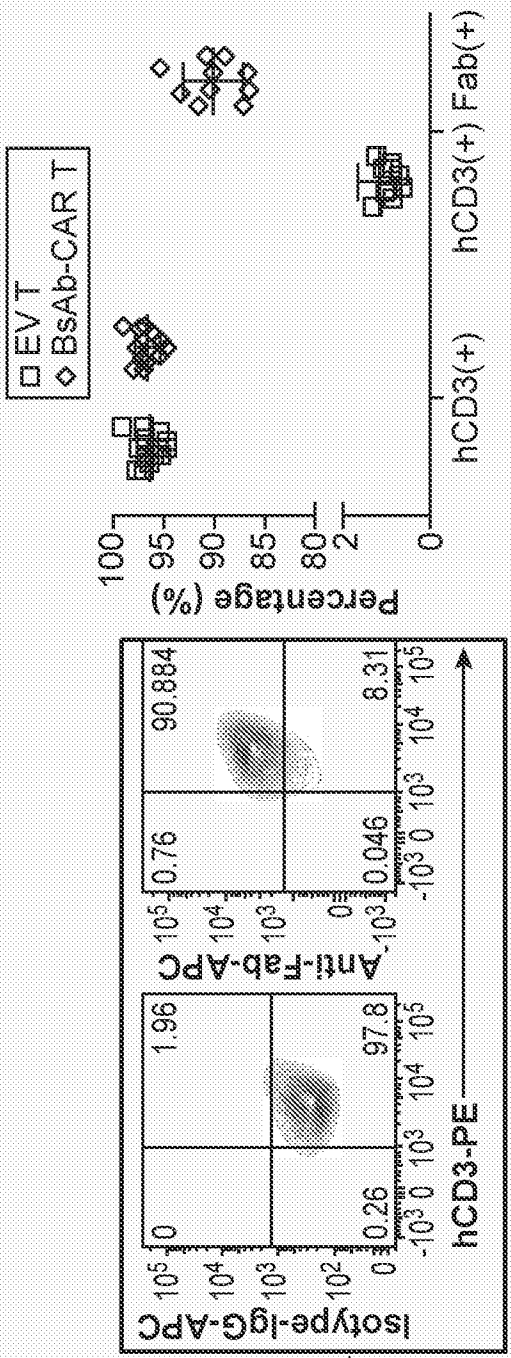



FIG. 21C

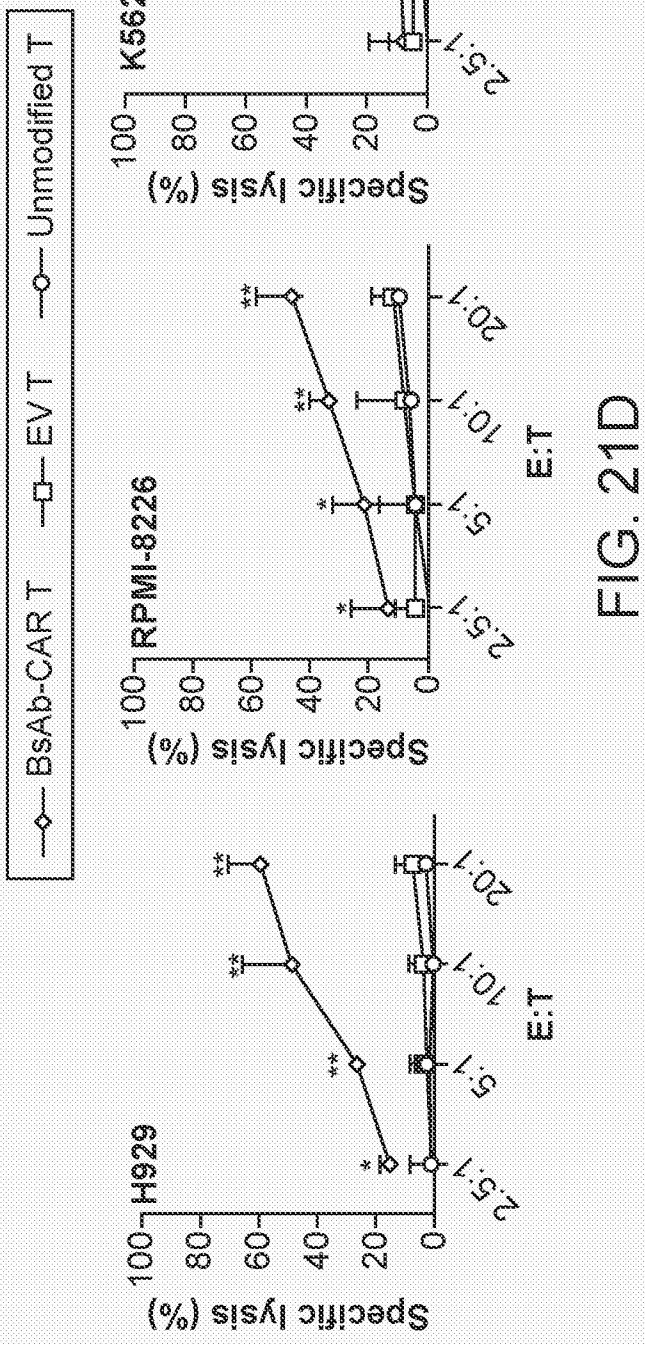



FIG. 21D

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/022639

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 35/17; A61K 39/00; A61P 35/00; C07K 16/28; C12N 5/10 (2019.01)
CPC - A61K 35/17; A61K 38/17; A61K 2039/507; C07K 14/7051; C07K 2319/00 (2019.05)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/93.21; 424/93.71; 530/387.3; 530/387.1; 536/23.4 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>--<br>Y | WO 2016/154585 A1 (SENTMAN et al) 29 September 2016 (29.09.2016) entire document | 1<br>-----<br>2, 3 |
| Y | WO 2016/154055 A1 (BLUEBIRD BIO, INC.) 29 September 2016 (29.09.2016) entire document | 2, 3 |
| A | WO 2017/024131 A1 (AVIDBIOTICS CORP.) 09 February 2017 (09.02.2017) entire document | 1-3 |
| A | US 2016/0046724 A1 (BROGDON et al) 18 February 2016 (18.02.2016) entire document | 1-3 |
| A | WO 2018/039247 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 01 March 2018 (01.03.2018) entire document | 1-3 |
| A | WO 2017/079705 A1 (JUNO THERAPEUTICS, INC.) 11 May 2017 (11.05.2017) entire document | 1-3 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 16 May 2019 | 13 JUN 2019 |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, VA 22313-1450<br>Facsimile No. 571-273-8300 | Authorized officer<br>Blaine R. Copenheaver<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/022639

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 28
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 28 has been held as an omnibus claim, as it refers to an invention "as disclosed herein".

3. ☒ Claims Nos.: 4-27
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.