

COMPOUND CHIMERIC ANTIGEN RECEPTOR (cCAR) TARGETING MULTIPLE ANTIGENS, COMPOSITIONS AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 62/571,608, filed October 12, 2017; and U.S. Provisional Patent Application No. 62/628,973, filed February 10, 2018. All of which are hereby incorporated by reference in their entirety.

BACKGROUND

T cells, a type of lymphocyte, play a central role in cell-mediated immunity. They are distinguished from other lymphocytes, such as B cells and natural killer cells (NK cells), by the presence of a T-cell receptor (TCR) on the cell surface. T helper cells, also called CD4+ T or CD4 T cells, express CD4 glycoprotein on their surface. Helper T cells are activated when exposed to peptide antigens presented by MHC (major histocompatibility complex) class II molecules. Once activated, these cells proliferate rapidly and secrete cytokines that regulate immune response. Cytotoxic T cells, also known as CD8+ T cells or CD8 T cells, express CD8 glycoprotein on the cell surface. The CD8+ T cells are activated when exposed to peptide antigens presented by MHC class I molecules. Memory T cells, a subset of T cells, persist long term and respond to their cognate antigen, thus providing the immune system with “memory” against past infections and/or tumor cells.

T cells can be genetically engineered to produce special receptors on their surface called chimeric antigen receptors (CARs). CARs are proteins that allow the T cells to recognize a specific protein (antigen) on tumor cells. These engineered CAR T cells are then grown in the laboratory until they number in the billions. The expanded population of CAR T cells is then infused into the patient.

Clinical trials to date have shown chimeric antigen receptor (CAR) T cells to have great promise in hematologic malignancies resistant to standard chemotherapies. Most notably, CD19-specific CAR (CD19CAR) T-cell therapies have had remarkable results including long-term remissions in B-cell malignancies (Kochenderfer, Wilson et al. 2010, Kalos, Levine et al. 2011,

Porter, Levine et al. 2011, Davila, Riviere et al. 2013, Grupp, Frey et al. 2013, Grupp, Kalos et al. 2013, Kalos, Nazimuddin et al. 2013, Kochenderfer, Dudley et al. 2013, Kochenderfer, Dudley et al. 2013, Lee, Shah et al. 2013, Park, Riviere et al. 2013, Maude, Frey et al. 2014).

Despite the success of CAR therapy in B-cell leukemia and lymphoma, the application of CAR therapy to soft tissue tumors has not yet been well established. Given that malignant soft tissue tumor are associated with dramatically poorer outcomes compared to those of B-cell malignancies (Abramson, Feldman et al. 2014), CAR therapy in this respect has the potential to further address a great clinical need.

There are some roadblocks that hinder the broader adoption of CAR therapeutic approach. Among the most general challenges are: (1) selection of antigen target and chimeric antigen receptor(s); (2) CAR design; (3) tumor heterogeneity, particularly the variance in the surface expression of tumor antigens. Targeting single antigen carries the risk of immune escape and this could be overcome by targeting multiple desired antigens.

Most CAR chimeric antigen receptors are scFvs derived from monoclonal antibodies and some of these monoclonal antibodies have been used in the clinical trials or treatment for diseases. However, they have limited efficacy, which suggests that alternative and more potent targeting approaches, such as CARs are required.

Target discovery and selection are the initial step as there are no general rules to ensure or guide CAR design that are efficacious.

scFvs are the most commonly used chimeric antigen receptor for CARs. However, CAR affinity binding and locations of the recognized epitope on the antigen could affect the function. Additionally the level of the surface CAR expression on the T cells or NK cells is affected by an appropriate leader sequence and promoter. However, overexpressed CAR proteins could be toxic to cells.

Therefore, there remains a need for improved chimeric antigen receptor-based therapies that allow for more effective, safe, and efficient targeting of T-cell associated malignancies

Furthermore, CAR targeting neuroblastoma is quite challenging because of the presence of heterogeneous tumor populations as well the presence of tumor micro-environment suppression. Antigen-specific immunotherapies for neuroblastoma have long been pursued to improve the patient treatment outcomes but success thus far has been limited as many these therapies have either been ineffective in the clinic or have an uncertain impact on patient

outcomes. The ideal target(s) in neuroblastoma or other soft tissue tumors (such as sarcomas), diseases of great antigenic diversity, has not been established. The identification of appropriate target (s) is an important step for the CAR design and the CAR design is required to address tumor heterogeneity, CAR persistency and tumor microenvironment suppression. There is no general rule that CAR design is efficacious and safe.

Therefore, there remains a need for improved chimeric antigen receptor-based therapies that allow for more effective, safe, and efficient targeting of soft tissue tumors.

SUMMARY

In one aspect, the present disclosure refers to an ex vivo engineered T cell or NK cell co-expressing two distinct chimeric antigen receptor (CAR) units at the cell surface, wherein the engineered T cell or NK cell comprises a nucleotide sequence comprising from 5' to 3' a first polynucleotide encoding a first chimeric antigen receptor polypeptide (first CAR), a second polynucleotide encoding a second chimeric antigen receptor polypeptide (second CAR), a nucleotide encoding a first viral self-cleavage peptide disposed between the first CAR and second CAR, under the transcriptional control of a single promoter, wherein: (i.) the first CAR polypeptide comprises a first antigen recognition domain selected from the group consisting of CS-1, CD5, CD19, CD20, CD123, BCMA, CD38, CLL-1, and CD33; a first signal peptide; a first hinge region; a first transmembrane domain; a first co-stimulatory domain; and a first signaling domain; and (ii.) the second CAR comprises a second antigen recognition domain selected from the group consisting of CS-1, CD5, CD19, CD20, CD123, BCMA, CD38, CLL-1, and CD33; a second signal peptide; a second hinge region; a second transmembrane domain; a second co-stimulatory domain; and a second signaling domain; wherein the first antigen recognition domain and the second antigen recognition domain are different; wherein the engineered T cell or NK cell comprises an enhancer selected from the group consisting of IL-15/IL-15sushi, IL-15/IL-15 sushi anchor, 4-1BBL, and IL-15, wherein the engineered T cell or NK cell comprises SEQ ID NO. 42 (a BCMA-CS 1 compound chimeric antigen receptor (cCAR) polypeptide and IL-15/IL-15 sushi); SEQ ID NO. 34 (a CD123-CD33 cCAR polypeptide and IL-15/IL-15 sushi); SEQ ID NO. 60 (a CLL1-CD33 cCAR polypeptide and IL-15/IL-15sushi); SEQ ID NO. 40 (a BCMA-CD38 cCAR polypeptide, 4-1BBL and IL-15/IL-15sushi); SEQ ID NO. 36 (CD123-CLL1 cCAR polypeptide, and IL-15/IL-15sushi); SEQ ID NO. 28 (a CD20-CD19 cCAR

polypeptide, and IL-15/IL-15sushi; or SEQ ID NO. 50 (CD269-A7D-L-15/IL-15sushi anchor-2G), and wherein said enhancer is separated from the first CAR and second CAR by a nucleotide encoding a second viral self-cleavage peptide that flanks either end of the two distinct CAR units.

In yet another aspect, the present disclosure refers to the use of an engineered cell as described herein in the manufacture of a medicament for treating a cell proliferative disease, wherein

the medicament is to be administered to a patient in need thereof, and wherein the cell proliferative disease comprises a T-cell malignancy, leukemia, or a lymphoma.

In one example, the present disclosure provides an engineered cell having a first chimeric antigen receptor polypeptide including a first antigen recognition domain, a first signal peptide, a first hinge region, a first transmembrane domain, a first co-stimulatory domain, and a first signaling domain; and a second chimeric antigen receptor polypeptide including a second antigen recognition domain, a second signal peptide, a second hinge region, a second transmembrane domain, a second co-stimulatory domain, and a second signaling domain; wherein the first antigen recognition domain is different than the second antigen recognition domain, and the first antigen recognition domain and second antigen rejection domain are selected from the group consisting of interleukin 6 receptor, NY-ESO-1, alpha fetoprotein (AFP), glypican-3 (GPC3), BAFF-R, BAFF, APRIL, BCMA, TACI, LeY, CD5, CD4, CD3, CD2, CD52, GD2, CD13, CD14, CD15 CD19, CD20, CD22, CD33, CD30, CD41, CD45, CD61, CD64, CD68, CD117, CD123, CD138, CD267, CD269, CD38, Flt3 receptor, CD4, CLL-1 and CS1(SLAMF7).

In another example, the present disclosure provides an engineered polypeptide including a chimeric antigen receptor and an enhancer.

In another example, the present disclosure provides a method of reducing the number of target cells including the steps of (i.) contacting said target cells with an effective amount of an engineered cell having at least one chimeric antigen receptor polypeptide, for engineered cells having multiple chimeric antigen receptor polypeptides, each chimeric antigen receptor polypeptide is independent; and (ii.) optionally, assaying for the reduction in the number of said cells. The target cells include at least one cell surface antigen selected from the group consisting of GD2, GD3, , ROR1, PSMA, PSCA (prostate stem cell antigen), MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, MUC1, MUC2, MUC3, MUC4, MUC5, CD30, EGFRvIII, CD33, CD123, CLL-1, immunoglobulin kappa and lambda, CD38, CD52, CD19, CD20, CD22, CD38, BCMA, CS1, NKG2D receptor, April receptor, BAFF receptor, TACI, CD3, CD4, CD8, CD5, CD7, CD2, and CD138. The target antigens can also include viral or fungal antigens, such as E6 and E7 from the human papillomavirus (HPV) or EBV (Epstein Barr virus) antigens.

In another example, the present disclosure provides methods for treating B-cell lymphoma, T-cell lymphoma, multiple myeloma, chronic myeloid leukemia, acute myeloma leukemia, myelodysplastic syndromes, chronic myeloproliferative neoplasms, B-cell acute

lymphoblastic leukemia (B-ALL), blastic plasmacytoid dendritic cell neoplasm, lung cancer, liver cancer, brain cancer, osteosarcoma, breast cancer, prostate cancer and cell proliferative diseases by administering any of the engineered cells described above to a patient in need thereof.

In another example, the present disclosure provides a method of treating an autoimmune disease, said method including administering an engineered cell as described herein to a patient in need thereof; wherein said autoimmune disease comprises systemic lupus erythematosus (SLE), multiple sclerosis (MS), Inflammatory bowel disease (IBD), Rheumatoid arthritis, Sjögren syndrome, dermatomyositis, autoimmune hemolytic anemia, Neuromyelitis optica (NMO), NMO Spectrum Disorder (NMOSD), idiopathic thrombocytopenic purpura (ITP), antineutrophil cytoplasmic autoantibodies (ANCAs) associated with systemic autoimmune small vessel vasculitis syndromes or microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA, Wegener's granulomatosis), or eosinophilic granulomatosis with polyangiitis (EGPA, Churg-Strauss syndrome) and TTP (thrombotic thrombocytopenic purpura)

The present disclosure provides chimeric antigen receptors (CARs) targeting non-hematologic malignancies, compositions and methods of use thereof.

In one example, the present disclosure provides an engineered cell having a first chimeric antigen receptor polypeptide including a first antigen recognition domain, a first signal peptide, a first hinge region, a first transmembrane domain, a first co-stimulatory domain, and a first signaling domain; and a second chimeric antigen receptor polypeptide including a second antigen recognition domain, a second signal peptide, a second hinge region, a second transmembrane domain, a second co-stimulatory domain, and a second signaling domain; wherein the first antigen recognition domain is different than the second antigen recognition domain.

In another example, the present disclosure provides an engineered polypeptide including a chimeric antigen receptor and an enhancer (s). In a further example, an enhancer can be selected from at least one of the group including, but not limited, IL-2, IL-7, IL-12, IL-15, IL-15/IL-15sush, IL-15/IL-15sushi anchor, IL-15/IL-15RA, IL-18, IL-21, IL-21 anchor, PD-1, PD-L1, CSF1R, CTAL-4, TIM-3, cytoplasmic domain of IL-15 receptor alpha, 4-1BBL, IL-21, IL-21 anchor and TGFR beta, receptors.

In some examples, CAR having an antigen recognition domain (s) is part of an expression cassette. In a preferred example, the expressing gene or the cassette may include an

accessory gene or a tag or a part thereof. The accessory gene may be an inducible suicide gene or a part thereof, including, but not limited to, caspase 9 gene. The “suicide gene” ablation approach improves safety of the gene therapy and kills cells only when activated by a specific compound or a molecule. In some examples, the epitope tag is a c-myc tag, CD52, streptavidin-binding peptide (SBP), truncated EGFR gene (EGFRt) or a part or a combination thereof.

In some examples, CAR cells can be ablated by administering an anti-CD52 monoclonal antibody (CAMPATH) to a subject.

In another example, the present disclosure provides methods for treating soft tissue tumors, carcinoma, sarcomas, leukemia, and cell proliferative diseases by administering any of the engineered cells described above to a patient in need thereof.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: CAR construction and expression

(A) Two discrete CAR units: an anti-BCMA CAR comprised of: a CD8-derived hinge (H) and transmembrane (TM) regions, and 4-1BB co-activation domains linked to the CD3 ζ signaling domain is fused to a complete anti-CS1 CAR by a self-cleaving P2A peptide. A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the BC1cCAR (BCMA-CS1 cCAR) molecule on the T-cell surface. (B) Expression of BC1cCAR was measured by FACS against control T-cells. BCMA also called CD269.

Figure 2: In vitro evaluation of BC1cCAR T-cells against myeloma cell lines

(A) BC1cCAR and control T-cells cultured with MM1S and RPMI-8226 cells for 24 hours at E:T ratios of 2:1 and 5:1. Target cells were stained by Cytotracker dye (CMTMR) to distinguish them from effector T-cells, and are indicated in red. Populations were gated by BCMA, CS1, and CMTMR. (B) BC1cCAR and control T-cells were incubated with U266 (BCMA⁺CS1^{dim}) cells under similar conditions. (C) Graphical summary of BC1cCAR T-cell in vitro cytotoxicity against various myeloma cell lines.

Figure 3: Primary patient cell phenotypes

Primary cells were assayed by FACS for BCMA and CS1 expression. Density plots represent major antigen populations.

Figure 4: Characterization of BC1cCAR T-cell anti-tumor activity against primary myeloma tumor cells

(A) Co-cultures against BCMA⁺CS1⁺ primary myeloma cells (MM7-G) were carried out over 24 hours and target cells pre-stained with CMTMR. Populations were gated by BCMA and CS1, along with CMTMR, and flow cytometry plots show target tumor populations in red (left). Bar graph summarizing in vitro cytotoxicity (right). (B) Co-cultures with MM10-G primary cells were conducted under similar conditions. BCMA⁺CS1⁺ double positive populations (purple) and CS1⁺ only populations (dark blue) by FACS. Specific cytotoxicity summarized (below). (C) BCMA^{dim}CS1^{dim} primary cells (MM11-G) show BC1cCAR anti-tumor activity over a range of E:T dosages. (D) Summary panel graph showing results of BC1cCAR in vitro screening.

Figure 5: Functional validation of BC1cCAR antigen specificity

(A) A CML cell line (K562) was transduced to stably express either BCMA or CS1. Histogram population shifts in their respective antigen expression ranges show expression. (B) Short term (4 hour – 8 hour) cultures of BC1cCAR T-cells against either BCMA-K562 or CS1-K562 show antigen specific cytotoxicity correlating with E:T dosage increase. Wild-type K562 cells were

used as a negative control. A CS1 single CAR (red bar) was generated to compare efficacy with BC1cCAR against CS1-K562 cells. (C) Long-term cultures (48 hours) conducted with a 1:1 mixture of BCMA-K562 cells and CS1-K562 cells. BC1cCAR, CS1-CAR, BCMA-CAR, and control T-cells were added at a 5:1 E:T ratio to each treatment well. Histogram plots showing residual populations (% gated) of BCMA or CS1 cells are shown per treatment condition, with red lines demarcating T-cell or target tumor populations.

Figure 6: Long-term sequential killing assay and tumor re-challenge

(A) Assay was conducted over a period of 168 hours without exogenous cytokines and initial culture was performed using a 1:1 E:T ratio of CAR cells or control cells mixed with BCMA⁺CS1⁺ MM1S cells. After 48 hours, flow cytometry analysis was acquired for a small sample collection and MM1S cells were re-introduced into each treatment well. Repeated through the 168 hour time-point. (B) T-cell proliferation and response after 48 hours. Images were taken on the day of flow cytometry acquisition and cells were stained with anti-BCMA, anti-CS1, and anti-CD3 antibodies, MM1S cells (circled, blue). (C) Similar image acquisition and FACS analysis was performed at the 108 hour time mark.

Figure 7: BC1cCAR T-cells demonstrate anti-leukemic effects in vivo.

(A) MM1S model tumor generated by injection of 1.0×10^6 luciferase⁺ cells per mouse. Mice treated with either BC1cCAR T-cells (right) or control T-cells (left) and IVIS image acquisition. (B) Average light intensity measured for BC1cCAR T-cell treated mice (red) compared to control T-cell treated mice (black). (C) Survival outcomes for BC1cCAR (red) and control (black) groups.

Figure 8: BC1cCAR T-cells exhibit improved cytotoxic effect in a mixed antigen xenogeneic mouse model

(A) Mouse model injected with BCMA and CS1 expressing K562 cells in a ratio of 4:1 BCMA:CS1 K562 cells (n=5 for each group). Mice were treated with either BC1cCAR T-cells, control T-cells, or a BCMA-specific CAR. Tumor burden was visualized by IVIS and plotted as a function of fluorescence intensity (right) for all groups. (B) Survival outcomes for control treated (black), BCMA-CAR treated (blue), and BC1cCAR (red) treated mice.

Figure 9: Improved BC1cCAR T-cell persistency and maintenance of tumor suppression in separate antigen models

(A) Whole blood samples from mice injected with either BCMA-K562 or CS1-K562 tumor cells (n=5 per group) were taken at time of sacrifice. Histogram population of BCMA or CS1 positive peaks represent tumor presence. (B) Aggregate tissue analysis of both whole blood and liver samples across sacrificed mice are summarized. Mice tumor cell counts were established by FACS of antigen positive cells per 250000 cells collected per sample and averaged across all mice per treatment group. (C) Whole blood and liver tissues were also analyzed for T-cell persistency by CD3 expression at time of sacrifice, summarized across all sacrificed mice (right).

Figure 10A: Analysis of mouse whole blood from separately injected BCMA-K562 or CS1-K562 injected mice

At times of sacrifice (various), mice whole blood was collected and labeled with antibodies against CD3, CD45, BCMA, and CS1. Histograms were constructed to visualize presence of tumor and counts were averaged across 250 000 events to generate graphical summaries. Some mice died before sacrifice, and were unusable for sample collection.

10B

Figure 10B: Analysis of mouse liver from separately injected BCMA-K562 or CS1-K562 injected mice

At times of sacrifice (various), mice liver samples were collected and labeled with antibodies against CD3, CD45, BCMA, and CS1. Histograms were constructed to visualize presence of tumor and counts were averaged across 250 000 events to generate graphical summaries. Some mice died before sacrifice, and were unusable for sample collection.

Figure 11A. A schematic representation of cCAR-T with IL-15/IL-15sushi enhancer construct. The construct comprises a SFFV promoter driving the expression of multiple modular units of CARs, and IL-15/IL-15sushi linked by P2A and T2A peptide respectively. Upon cleavage of the linker, the cCARs split and engage upon targets expressing BCMA and/or CS1 and a secreting enhancer fusion of IL-15/IL-15sushi. As a novel cCAR construct, the activation domains of the construct may include, but is not limited to, 4-1BB or CD28 on the BCMA CAR segment and a CD28 or 4-1BB on the CS1 CAR segment. The peptide self cleavage peptides of

the construct may include, but is not limited to, P2A, T2A, F2A and E2A. The secreting enhancer (s) of the construct may also include, but is not limited to, IL-15/IL-15sush, IL-15, IL-21, IL-18, IL-7, IL-21, and IL-12. The secreting enhancer, such as IL-15/IL-15sushi enhances CAR T or NK cell expansion and persistency. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion can also enhance CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

Figure 11B. Expression of CAR T cells. Buffy coat cells were activated 3 days with anti-human CD3 antibody. Cells were transduced with either control vector (left) and CD269-A7D-hu63-IL15/IL-15sushi (right). After 3 days of incubation, cells were harvested and labeled for flow cytometry. CAR T cells are represented as green dots (circled).

Figure 11C. CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cells specifically lyse the K562 tumor cell line, which is synthetically expressing either BCMA (left) or CS1 (right) surface antigen, in co-culture assays. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 48 hours and were directly analyzed by flow cytometry for anti-human CS1 (CD319) and CD3.

Figure 11D. CD269-A7D-CS1-IL15/IL15sushi CAR T cells demonstrate strong anti-tumor effects *in vivo* against MM.1S tumor cell line (a myeloma cell line). NSG mice were sublethally irradiated and intravenously injected with 4.0×10^6 luciferase-expressing MM.1S cells (Day 0) to induce measurable tumor formation. Starting 8 days after injection of tumor cells, mice were intravenously injected with a course of 15×10^6 either CD269-A7D-CS1-IL15/IL15sushi CAR T or vector control T cells. On days 8 (before T cell injection) and 12 (72 hours after T cell injection), mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging.

Figure 11E. CD269-A7D-CS1-hu63-IL15/IL15sushi NK cells express functional IL15. NK-92 cell line was transduced with lentiviral vector containing CD269-A7D-CS1-hu63-IL15/IL15sushi CAR. (A) Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype. (B) CD269-A7D-CS1-hu63-IL15/IL15sushi CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5×10^6 cells per mL, in 1 mL total volume.

Cells were added to duplicate wells; one well of each pair contained IL-2 at 300 IU/mL, the other well did not. After 48 hours (Day 2), cells were counted (B), and the volume increased to yield a concentration of approximately 0.5×10^6 cells/mL. This process was repeated on Days 4, 6 and 8.

5 **Figure 11F.** Sorted CD269-A7D-CS1-hu63-IL15/IL15sushi NK cells and wild-type control NK-92 cells were cultured in separate wells for 72 hours. Supernatant was collected and subjected to ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained on a plate reader were compared to a standard curve (A) generated with human IL-15 to determine concentration of IL-15 in the supernatants (B).

10 **Figure 12: Genetic structure and function of CD123b-CD33b cCAR**

(A) Representation of CD123-CD33cCAR. (B) CD123b-CD33b cCAR T-cells are created by the viral transduction of patient donor T-cells with the CD123b-CD33b cCAR gene construct. The translated CD123 and CD33 CAR proteins are then expressed on the surface of the CAR T-cells, where they can recognize and bind the CD123 and CD33 target proteins on the surface of
15 leukemic cells. The pharmacologic effect and mechanism of CD123b-CD33b cCAR T-cells is mediated by CD123b-CD33b cCAR recognition of the antigen, which triggers CD3zeta/Zap70 canonical cytotoxic T-cell activity further enhanced by the incorporation of CD28 or 4-1BB co-activation domains in the construct, creating a "second generation" CAR.

20 **Figure 13: CD123b-CD33b cCAR Transduction Efficiency**

Flow cytometry was used to determine CD123b-CD33b cCAR expression levels on the T-cell surface after transduction.

Figure 14: CD123b-CD33b cCAR T-cells demonstrate targeted lysis of MOLM13 and U937 tumor cells lines.

25 (A) Flow cytometry analysis of control T-cells and CD123b-CD33b cCAR T-cells against MOLM13 (an AML cell line) tumor target cells at 2:1 and 5:1 E:T ratios. The target cell population is encircled. (B) Flow cytometry analysis of control T-cells and CD123b-CD33b cCAR T-cells against U937 tumor target cells at 2:1 and 5:1 E:T ratios. The target cell population is encircled. (C) MOLM13 tumor cells (CD123+CD33+) and U937 cells (CD123-
30 CD33+) alone stained for markers and their percent lysis summary at both E:T ratios. (D) Dose-

dependent cultures performed with HL60 (CD123dimCD33+) and KG1a (CD123dimCD33+) cells display high cCAR killing efficiency at E:T ratios ranging from 0.25:1 to 10:1.

Figure 15: CD123b-CD33b cCAR T-cells demonstrate targeted lysis of primary patient tumor cells.

(A) Flow cytometry analysis of control T-cells and CD123b-CD33b cCAR T-cells against PT1 tumor target cells at 2:1 and 5:1 E:T ratios. The target cell population is encircled. (B) Flow cytometry analysis of control T-cells and CD123b-CD33b cCAR T-cells against PT2 tumor target cells at 2:1 and 5:1 E:T ratios. The target cell population is encircled. (C) Flow cytometry analysis of control T-cells and CD123b-CD33b cCAR T-cells against PT3 tumor target cells at 2:1 and 5:1 E:T ratios. The target cell population (CD123+CD34+) is encircled and further broken down by CD38 expression to display LSC (CD123+CD34+CD38-) elimination. (D) Flow cytometry analysis of control T-cells and CD123b-CD33b cCAR T-cells against PT4 tumor target cells at 2:1 and 5:1 E:T ratios. The target cell population (CD33+ bulk disease) is encircled. (E) Percent lysis summary of CD123b-CD33b cCAR T-cells against all four patient samples at both 2:1 and 5:1 E:T ratios.

Figure 16: CD123b-CD33b cCAR T-cells ablate cells expressing either the CD33 or CD123 antigen with high efficacy.

(A) Flow cytometry analysis of control T-cells and CD123b-CD33b cCAR T-cells against wild-type (WT) Jurkat tumor cells and Jurkat cells expressing CD123 (Jurkatxp123) at a 2:1 E:T ratio. The target cell population is encircled. (B) Flow cytometry analysis of control T-cells and CD123b-CD33b cCAR T-cells against wild-type (WT) Jurkat tumor cells and Jurkat cells expressing CD33 (Jurkatxp33) at a 2:1 E:T ratio. The target cell population is encircled. (C) Percent lysis summary of CD123b-CD33b cCAR T-cells against WT Jurkat cells, Jurkat xp33, and Jurkat xp123 cells at a 2:1 E:T ratio.

Figure 17: CD123b-CD33b cCAR T-cells demonstrate a profound anti-leukemic effect against MOLM13 and U937 cell lines in two in vivo xenograft mouse models.

(A) IVIS imaging of luciferase-expressing MOLM13 cells on days 3, 6, 9, and 13 allowing tumor burden visualization (n=8 for each group). Graphical representation of tumor burden

comparison between CD123b-CD33b cCAR T-cell and control T-cell treated mice over time. Tumor reduction is statistically significant from day 6 onward. Kaplan-Meier survival analysis curve represents survival outcomes (Mantel-Cox log-rank test $p = 0.0082$). (B) IVIS imaging of luciferase-expressing U937 cells on days 3, 6, 9, and 13 allowing tumor burden visualization (n=8 for each group). Graphical representation of tumor burden comparison between CD123b-CD33b cCAR T-cell and control T-cell treated mice over time. Tumor reduction is statistically significant from day 6 onward. Kaplan-Meier survival analysis curve represents survival outcomes (Mantel-Cox log-rank test $p = 0.0082$). (C) Peripheral blood of MOLM13 and U937 mice tumor models. Flow cytometry allowed visualization of CD45+CD3+ T-cells and CD45+CD33+ tumor cells.

Figure 18: Depletion of infused CD123b-CD33b cCAR T-cells following treatment with CAMPATH.

(A) Experimental schema to evaluate the effect of CAMPATH administration after CD19b-CD123 cCAR T-cell infusion into NGS mice. 10×10^6 CD19b-CD123 cCAR T-cells were injected intravenously into sublethally irradiated mice (n=6) and ~24 hours later, CAMPATH (0.1mg/kg) or PBS were intraperitoneally injected (n=3 of each, except for hour 6 where n=2 for control group). 6 and 24 hour later, peripheral blood was collected to determine the persistence of CAR T-cells. (B) Representation of persistence of infused CD19b-CD123 cCART-cells in peripheral blood 6 hours later with or without CAMPATH treatment. Presence of CD19b-CD123 cCART-cells was detected by flow cytometry. (C) Representation of persistence of infused CD19b-CD123 cCART-cells in peripheral blood 24 hours later with or without CAMPATH treatment. Presence of CD19b-CD123 cCAR T-cells was detected by flow cytometry.

Figure 19: Structure organization of CD19b-CD123 cCAR

A schematic representation of cCAR-T construct (CD19b-CD123cCAR). The construct comprises a SFFV promoter driving the expression of-multiple modular units of CARs linked by a P2A peptide. Upon cleavage of the linker, the cCARs split and engage upon targets expressing CD19b CAR and CD123 CAR targeting CD19 and CD123 antigen respectively . As a novel cCAR construct, the activation domains of the construct may include, but is not limited to, 4-1BB on the CD19b CAR segment and a CD28 region on the CD123 CAR. A hinge domain (H),

a transmembrane domain (TM), a co-stimulatory domain (CD28 or 4-1BB) and the intracellular signaling domain CD3 zeta (CD3).

Figure 20: Transduction efficiency of CD19b-CD123 cCAR

- 5 Activated T cells were transduced with thawed lentivirus expressing CD19b-CD123 cCAR on retronectin-coated plates. After transduction, cells are washed and expanded; flow analysis (F(Ab')₂ labeling) is done to confirm CAR efficiency.

Figure 21: CD19b-CD123 cCAR T cells demonstrate specific and efficacious lysis of CD19+ and CD123+ leukemia/lymphoma cell lines.

- 10 (A) Flow cytometry analysis of control T-cells and CD19b-CD123 cCAR T-cells against artificially-induced CD19+ K562 cells and control K562 cells at 5:1 E:T ratios at 16 and 48 hours. The target cell population is depicted in red. Non-transduced CD19- cells are depicted in dark yellow. (B) Flow cytometry analysis of control T-cells and CD19b-CD123 cCAR T-cells against artificially-induced CD19+ K562 cells and control K562 cells at 5:1 E:T ratios at 16
15 hours. The target cell population is depicted in red. Non-transduced CD123- Jurkat cells are depicted in purple. (C) Flow cytometry analysis of KG1a tumor cells (CD123+CD19-) and SP53 cells (CD123-CD19+) at 5:1 E:T ratio, at 16 and 48 hours. (D) Summary graph of tumor cell percent lysis.

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Figure 22: CD19b-CD123 cCAR T cells demonstrate targeted lysis of primary patient cells.

- (A) Flow cytometry analysis of PT1 and PT2 tumor cell phenotypes. (B) Flow cytometry analysis of control T-cells and CD19b-CD123 cCAR T-cells against PT1 tumor target cells a 5:1 E:T ratio, at 24 hours. The target cell population is depicted in red. (C) Flow cytometry analysis
25 of control T-cells and CD19b-CD123 cCAR T-cells against PT2 tumor target cells a 5:1 E:T ratio, at 24 and 48 hours. The target cell population is depicted in red. (D) Percent lysis summary of CD19b-CD123 cCAR T-cells against patient samples at a 5:1 E:T ratio at 24 and 48 hours.

Figure 23: CD19b-CD123 cCAR T-cells demonstrate a profound anti-leukemic effect against MOLM13 and REH cell lines in two in vivo xenograft mouse models.

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(A) IVIS imaging of luciferase-expressing MOLM13 cells on days 3, 6, 8, and 11 allowing tumor burden visualization (represented mice for each group). (B) Graphical representation of tumor burden comparison between CD19b-CD123 cCAR T-cell and control T-cell treated mice over time, tumor burden was measured both dorsally and ventrally. Tumor reduction is statistically significant from day 6 onward. (C) Kaplan-Meier survival analysis curve represents survival outcomes (Mantel-Cox log-rank test $p = 0.0031$). (D) IVIS imaging of luciferase-expressing REH cells on day 16, allowing for tumor burden visualization ($n=5$ for each group). (E) Graphical representation of tumor burden comparison between CD19b-CD123 cCAR T-cell and control T-cell treated mice over time. Tumor reduction is statistically significant. Tumor burden was measured dorsally and ventrally. (F) Kaplan-Meier survival analysis curve represents survival outcomes (Mantel-Cox log-rank test $p = 0.0016$).

Figure 24. A Link by P2A schematic showing CAR, 4-1BB and IL-21 in a single construct (CAR co-expressing IL-21) and its expression in T or NK cells.

The construct consists of a SFFV promoter driving the expression of CAR with costimulatory domain, 4-1BB). Upon cleavage of the linkers, a CAR and IL-21 split and engage upon targets expressing antigen. CAR T cells received not only costimulation through the 4-1BB or CD28 but also 4-1BB ligand (4-1BBL or CD137L) or IL-21. The CD3-zeta signaling domain complete the assembly of this CAR-T. The IL-21 signal peptide is replaced with IL-2 signal peptide for a better secretion of IL-21. H, CD8a hinge region, TM, CD8a transmembrane domain. Example of CAR with IL-21 can be CD19-IL-21 CAR, BCMA-IL-21 CAR, CD4-IL-21 CAR and CD45-IL-21 CAR.

Figure. 25. Schematic diagram to elucidate the construct (CAR co-expressing IL-21 anchor) and its expression in T or NK cells.

A CAR with IL-21 anchor is linked with the P2A self-cleaving sequence. The IL-21 anchor fusion is composed of IL-2 signal peptide fused to IL-21, and linked to CD8 hinge region and CD8 transmembrane domain. The combination of CAR and IL-21 fusion is assembled on an expression vector and their expression is driven by the SFFV promoter. The IL-21 signal peptide is replaced with IL-2 signal peptide for a better secretion of IL-21 and anchoring on the cell surface. Example of CAR with IL-21 anchor can be CD19-IL-21 anchor CAR, BCMA-IL-21 anchor CAR, CD4-IL-21 anchor CAR and CD45-IL-21 anchor CAR.

Figure 26. A Link by P2A schematic showing CAR, 4-1BB and IL-18 in a single construct (CAR co-expressing IL-18) and its expression in T or NK cells.

The construct consists of a SFFV promoter driving the expression of CAR with costimulatory domain, 4-1BB). Upon cleavage of the linkers, a CAR and IL-18 split and engage upon targets expressing antigen. CAR T cells received not only costimulation through the 4-1BB or CD28 but also 4-1BB ligand (4-1BBL or CD137L) or IL-21. The CD3-zeta signaling domain complete the assembly of this CAR-T. The IL-21 signal peptide is replaced with IL-2 signal peptide for a better secretion of IL-18. H, CD8a hinge region, TM, CD8a transmembrane domain. The CD3-zeta signaling domain complete the assembly of this CAR-T. Example of CAR with IL-18 can be CD19-IL-18 CAR, BCMA-IL-18 CAR, CD4-IL-18 CAR and CD45-IL-18 CAR.

Figure. 27. Schematic diagram to elucidate the construct (CAR co-expressing IL-18 anchor) and its expression in T or NK cells.

A CAR with IL-18 anchor is linked with the P2A self-cleaving sequence. The IL-18 anchor fusion is composed of IL-2 signal peptide fused to IL-18 and linked to CD8 hinge region and CD8 transmembrane domain. The combination of CAR and IL-18 anchor fusion is assembled on an expression vector without CD3 zeta chain, and their expression is driven by the SFFV promoter. The IL-18 signal peptide is replaced with IL-2 signal peptide for a better secretion of IL-18 and then anchoring on the cell surface. Example of CAR with IL-18 anchor can be CD19-IL-18 anchor CAR, BCMA-IL-18 anchor CAR, CD4-IL-18 anchor CAR and CD45-IL-18 anchor CAR.

Figure 28A. Expression of different versions of anti-BCMA CAR or cCAR T cells. Buffy coat cells were activated 3 days with anti-CD3 antibody.

Cells were transduced with either control vector (top left) or various CD269 CAR lentiviral supernatants. After 3 days of incubation, cells were harvested and labeled for flow cytometry.

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Figure 28B. Expression of different versions of BCMA-CS1 cCAR T cells.

Buffy coat cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (top left) or various CD269 cCAR lentiviral supernatants. After 3 days of incubation, cells were harvested and labeled for flow cytometry.

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Figure 29A. CD269-A7D-CD19b CAR T cells specifically lyse the K562 tumor cell line, which is synthetically expressing CD19 surface antigen (K-19), in co-culture assays. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 18 hours and were directly analyzed by flow cytometry for CD19 and CD3. Each assay consists of K-19 target cells alone (left), control T cells (center panels) and CD269-A7D-CD19b CAR T cells (right panels). K-19 cells are circled.

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Figure 29B. CD269-A7D-CD19b CAR T cells specifically lyse the K562 tumor cell line, which is synthetically expressing BCMA surface antigen (K-BCMA), in co-culture assays.

Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 18 hours and were directly analyzed by flow cytometry for CD269 and CD3. Each assay consists of K-BCMA target cells alone (left), control T cells (center panels) and CD269-A7D-CD19b CAR T cells (right panels). K-BCMA cells are circled.

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Figure 30A. Expression of different versions of BCMA-CS1 cCAR T cells.

Buffy coat cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (top left) or various CD269 (BCMA) cCAR lentiviral supernatants. After 3 days of incubation, cells were harvested and labeled for flow cytometry.

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Figure 30B. Expression of different versions of BCMA-CS1 cCAR T cells or enhanced BCMA CAR T cells.

Buffy coat cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (top left) or various CD269 (BCMA) CAR lentiviral supernatants. After 3 days of incubation, cells were harvested and labeled for flow cytometry.

Figure 30C. CD269-A7D-CD19b CAR T cells specifically lyse the K562 tumor cell line, which is synthetically expressing BCMA surface antigen (K-BCMA), in co-culture assays.

Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 18 hours and were directly analyzed by flow cytometry for CD269 and CD3. Each assay consists of K-BCMA target cells alone (left), control T cells (center panels) and CD269-A7D-CD19b CAR T cells (right panels). K-BCMA cells are circled.

Figure 30D. CD269-A7D-CD19b CAR T cells specifically lyse the K562 tumor cell line, which is synthetically expressing CD19 surface antigen (K-19), in co-culture assays.

Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 18 hours and were directly analyzed by flow cytometry for CD19 and CD3. Each assay consists of K-19 target cells alone (left), control T cells (center panels) and CD269-A7D-CD19b CAR T cells (right panels). K-19 cells are circled. Results are summarized in the graph in the lower left. (N=2).

Figure 30E. Summary lysis of K562-BCMA (K-BCMA) and K562-CD19 (K-19) cells by CD269-A-7D-CD19b cCAR T cells.

Figure 30F. CD269-A7D cCAR T cells specifically lyse the MM1S tumor cell line in co-culture assays.

Co-culture experiments were performed at an effector to target ratio of 5:1 for 18 hours and were directly analyzed by flow cytometry for CD269 (BCMA) and CMTMR (CellTracker). Each assay consists of MM1S target cells alone (left), control T cells (top center panel), CD269-A7D-41BBL (bottom center), CD269-A7D-C11D (top right) and CD269-A7D-

CS1-hu63 cCAR T cells (bottom right). MM1S cells are represented by blue dots. (N=2).

Figure 30G. Different versions of CD269-CS1 cCAR or enhanced CD269 CAR T cells specifically lyse the K562-BCMA tumor cell line in co-culture assays.

5 Co-culture experiments were performed at an effector to target ratio of 5:1 for 18 hours and were directly analyzed by flow cytometry for CD269 and CD3. Each assay consists of MM1S target cells alone (left), control T cells (top center panel), CD269-A7D-41BBL (bottom center), CD269-A7D-C11D (a cCAR targeting two different epitopes of BCMA antigen) (top right) and CD269-A7D-CS1-hu63 CAR T cells (bottom right). K-BCMA cells are represented by green dots. (N=2).

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Figure 30H. CD269-A7D-CS1-hu63 CAR T cells specifically lyse the K562-CS1 tumor cell line in co-culture assays, while CD269-A7D-C11D cCAR (a cCAR targeting different epitopes of BCMA antigen, without a CS1 CAR) do not. Co-culture experiments were performed at an effector to target ratio of 5:1 for 18 hours and were directly analyzed by flow cytometry for CD269 and CD3. Each assay consists of MM1S target cells alone (left), control T cells (center panel), CD269-A7D-C11D (top right) and CD269-A7D-CS1-hu63 CAR T cells (bottom right). K-CS1 cells are represented by dark green dots. (N=2).

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20 **Figure 30I. Summary lysis of MM1S myeloma cells by CD269-A7D-41BBL, CD269-A7D-C11D and CD269-CS1-hu63 CAR T cells.**

Figure 30J. Summary lysis of K-BCMA (K562 expressing BCMA) cells by CD269-A7D-41BBL, CD269-A7D-C11D and CD269-CS1-hu63 CAR T cells.

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Figure 30K. Summary lysis of K-CS1 (K562 expressing CS1) cells by CD269-A7D-C11D and CD269-CS1-hu63 cCAR T cells.

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Figure 31. Expression of CLL1-CD33b CAR T cells. Buffy coat cells were activated 3 days

with anti-CD3 antibody.

Cells were transduced with either control vector (left) or CLL1-CD33b CAR (right) lentiviral supernatant. After 3 days of incubation, cells were harvested and labeled for flow cytometry.

Figure 32A. CLL1-CD33b CAR T cells do not lyse REH tumor cell line in co-culture assays.

Target cells were prelabeled with CFSE dye to distinguish them from T cells. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 18 hours and were directly analyzed by flow cytometry for CFSE and CD3. Each assay consists of REH target cells alone (left), control T cells (center panels) and CLL1-CD33b CAR T cells (right panels). REH cells are represented as purple dots. Note: REH cells do not express CLL1 (CLL-1) or CD33.

Figure 32B. CLL1-CD33b CAR T cells do not lyse CCRF-CEM tumor cell line, in co-culture assays.

Target cells were prelabeled with CFSE dye to distinguish them from T cells. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 18 hours and were directly analyzed by flow cytometry for CFSE and CD3. Each assay consists of CCRF-CEM target cells alone (left), control T cells (center panels) and CLL1-CD33b CAR T cells (right panels). CCRF-CEM cells are represented as orange dots. Note: CCRF-CEM cells do not express CLL1 or CD33 antigen.

Figure 32C. CLL1-CD33b CAR T cells specifically lyse the Jurkat tumor cell line, which is synthetically expressing CLL-1 surface antigen in co-culture assays.

Target cells were prelabeled with CFSE dye to distinguish them from T cells. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 18 hours and were directly analyzed by flow cytometry for CFSE and CD3. Each assay consists of Jurkat-CLL1 (J-CLL) target cells alone (left), control T cells (center panels) and CLL1-CD33b CAR T cells (right panels). Jurkat-CLL cells are represented as blue dots.

Figure 32D. CLL1-CD33b CAR T cells specifically lyse the Jurkat tumor cell line, which is synthetically expressing CD33 surface antigen, in co-culture assays.

Target cells were prelabeled with CFSE dye to distinguish them from T cells. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 18 hours and were directly analyzed by flow cytometry for CFSE and CD3. Each assay consists of Jurkat-CD33 (J-33xp) target cells alone (left), control T cells (center panels) and CLL1-CD33b CAR T cells (right panels). Jurkat-CD33 (J-33xp) cells are represented as light blue dots.

Figure 32E. CLL1-CD33b cCAR T cells efficiently lyse HL60 tumor cell line in co-culture assays.

Target cells were prelabeled with CFSE dye to distinguish them from T cells. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 18 hours and were directly analyzed by flow cytometry for CFSE and CD3. Each assay consists of HL60 target cells alone (left), control T cells (center panels) and CLL1-CD33b CAR T cells (right panels). HL60 cells are represented as green dots.

Figure 32F. Summary of CLL1-CD33 cCAR (CLL-1-CD33 cCAR) lysis results in co-culture assays using different AML cell lines and Jurkat cells expressing either CLL-1 or CD33.

Figure 32G – CLL1-CD33b compound CAR T cells ablate HL60 target tumor cells

Cocultures were carried out overnight at E:T ratios of 2:1 and 5:1. Target HL60 cells mostly double positive for CLL-1 and CD33 were prelabeled with CFSE membrane dye. Flow cytometry acquisition (FACS) was conducted the next day using CD3, CLL-1, and CD33 antibodies.

Figure 32H – CLL1-CD33b compound CAR T cells ablate U937 target tumor cells

Cocultures were carried out overnight at E:T ratios of 2:1 and 5:1. Target U937 cells are highly positive for both CLL-1 and CD33 and were prelabeled with CFSE membrane dye. Flow cytometry acquisition (FACS) was conducted the next day using CD3, CLL-1, and CD33 antibodies.

Figure 32I – CLL1-CD33b compound CAR T cells minimally target negative control CCRF-CEM cells

Cocultures were carried out overnight at E:T ratios of 2:1 and 5:1. CCRF-CEM cells are

predominantly negative for CLL-1 and CD33 and were prelabeled with CFSE membrane dye. Flow cytometry acquisition (FACS) was conducted the next day using CD3, CLL-1, and CD33 antibodies.

5 **Figure 32J – *In vitro* summary of CLL1-CD33b compound CAR T cells against target cell lines**

All co-cultures were carried out overnight and target cells were prelabeled with CFSE membrane dye. Flow cytometry acquisition (FACS) was conducted the next day using CD3, CLL-1, and CD33 antibodies for all samples. Dose dependent co-cultures using HL60 target cells were
10 conducted in an escalating E:T ratio scheme under identical co-culture conditions.

Figure 32K – Antigen depletion by CLL1-CD33b compound CAR in relation to single CAR T cells in a mixed cell co-culture.

CD33 expressing and CLL-1 expressing Jurkat cells were produced by stable transfection of
15 CD33 or CLL-1 expressing cDNA into wild type Jurkat cells. Jurkat cells were then sorted for expression to establish homogeneous stable cell lines expressing either CD33 or CLL-1. For mixed cell co-culture, Jurkat cells expressing CD33 (Jurkat-CD33) and Jurkat cells expressing CLL-1 (Jurkat-CLL1) were mixed together in an approximate 1:1 ratio totaling 200,000 cells. Effector cells were then added in a 1:2 ratio (effector: target), totaling 100,000 T-cells in an
20 overnight culture. Flow cytometry acquisition (FACS) was conducted the next day using CD3, CLL-1, and CD33 antibodies for all samples. Histograms depicting antigen depletion under various CAR treatments are shown, with bars (left) depicting T-cell populations and antigen expressing Jurkat cells (right).

25 **Figure 32L – Summary of antigen depletion by CLL1-CD33b compound CAR in relation to single CAR T cells in a mixed cell co-culture.**

Graphs summarizing histogram data of the previous figure. Overall, CLL1-CD33b compound CAR T cells exhibit potent and targeted cytotoxicity against both CD33 and CLL-1 expressing Jurkat cells with ablation rates of greater than 85% against both cell types. Furthermore, CLL1-
30 CD33b compound CAR T cells were able to demonstrate superior cytotoxicity compared to a single anti-CD33b CAR T or a single anti-CLL-1 CAR T cell against their own respective

antigen populations. The compound CAR was able to target CD33 60% better than a CD33 CAR T and CLL-1 40% better than a CLL-1 CAR T cell.

Figure 33A. A Link by P2A schematic showing CD19 CAR and IL-21 in a single construct (Cd19 CAR co-expressing IL-21) and its expression in T or NK cells.

Figure 33B. Expression of CD19b-IL-21 CAR T cells and CD19-IL-21 anchor. Buffy coat cells were activated 3 days with anti-CD3 antibody.

Cells were transduced with either control vector (left), CD19b-IL-21, or CD19b-IL21-anchor CAR (right) lentiviral supernatant. After 3 days of incubation, cells were harvested and labeled for flow cytometry.

Figure 34. Schematic diagram to elucidate the construct (CD19 CAR co-expressing IL-21 anchor) and its expression in T or NK cells.

CD19 CAR with IL-21 anchor is linked with the P2A self-cleaving sequence. The IL-21 anchor fusion is composed of IL-2 signal peptide fused to IL-21, and linked to CD8 hinge region and CD8 transmembrane domain. The combination of CD19 CAR and IL-21 fusion is assembled on an expression vector and their expression is driven by the SFFV promoter. The IL-21 signal peptide is replaced with IL-2 signal peptide for a better secretion of IL-21 and anchoring on the cell surface.

Figure 35. A Link by P2A schematic showing BCMA CAR, and IL-18 in a single construct (BCMA CAR co-expressing IL-18) and its expression in T or NK cells.

The construct consists of a SFFV promoter driving the expression of CAR with costimulatory domain, 4-1BB). Upon cleavage of the linkers, BCMA CAR and IL-18 split and engage upon targets expressing antigen. CAR T cells received not only costimulation through the 4-1BB or CD28 but also 4-1BB ligand (4-1BBL or CD137L) or IL-18. The CD3-zeta signaling domain complete the assembly of this CAR-T. The IL-21 signal peptide is replaced with IL-2 signal peptide for a better secretion of IL-21. H, CD8a hinge region, TM, CD8a transmembrane domain.

Figure. 36. Schematic diagram to elucidate the construct BCMA (CAR co-expressing IL-18

anchor) and its expression in T or NK cells.

A CAR with IL-18 anchor is linked with the P2A self-cleaving sequence. The IL-18 anchor fusion is composed of IL-2 signal peptide fused to IL-18, and linked to CD8 hinge region and CD8 transmembrane domain. The combination of BCMA CAR and IL-18 anchor fusion is
 5 assembled on an expression vector and their expression is driven by the SFFV promoter. The IL-18 signal peptide is replaced with IL-2 signal peptide for a better secretion of IL-18 and anchoring on the cell surface.

Figure 37. A schematic representation of cCAR construct (BCMA-CD38 cCAR).

10 The construct comprises a SFFV promoter driving the expression of multiple modular units of CARs linked by a P2A cleavage peptide. Upon cleavage of the P2A linker, the cCARs split and engage upon targets expressing BCMA and/or CD38. Each unit of CAR bears a scFv against the antigen, a hinge domain (H), a transmembrane domain (TM), a co-stimulatory domain (including, but not limited to, CD28 or 4-1BB) and the intracellular signaling domain CD3 zeta
 15 chain. As a novel cCAR construct, the activation domains of the construct may include, but is not limited to, 4-1BB on the BCMA CAR segment and a CD28 region on the CD38 CAR.

Figure 38. A schematic representation of cCAR construct (BCMA1-BCMA2 cCAR).

The construct comprises a SFFV promoter driving the expression of multiple modular units of CARs linked by a P2A cleavage peptide. Upon cleavage of the P2A linker, the cCARs split and
 20 engage upon targets expressing BCMA1 (one of BCMA1 epitopes) and/or BCMA2 (another BCMA antigen epitope). Each unit of CAR bears a scFv against the antigen, a hinge domain (H), a transmembrane domain (TM), a co-stimulatory domain (including, but not limited to, CD28 or 4-1BB) and the intracellular signaling domain CD3 zeta chain. As a novel cCAR
 25 construct, the activation domains of the construct may include, but is not limited to, 4-1BB on the BCMA1 CAR segment and a CD28 region on the BCMA2 CAR. Each BCMA CAR targets its antigen epitope. The compound CAR, BCMA1-BCMA2 is able to recognize two distinct BCMA epitopes.

Figure 39A. A schematic representation of cCAR-T with IL-15/IL15sushi enhancer construct.

The construct comprises a SFFV promoter driving the expression of multiple modular units of
 30 CARs, and IL-15/IL-15sushi linked by P2A and T2A peptide respectively. Upon cleavage of the linker, the cCARs split and engage upon targets expressing CLL-1 and/or CD33 and a

secreting enhancer fusion of IL-15/IL-15sushi. As a novel cCAR construct, the activation domains of the construct may include, but is not limited to, 4-1BB or CD28 on the CLL-1 CAR segment and a CD28 or 4-1BB region on the CD33B CAR segment. The peptide self cleavage peptides of the construct may include, but is not limited to, P2A, T2A, F2A and E2A. The secreting enhancer (s) of the construct may also include, but is not limited to, IL-15/IL-15sushi, IL-15, IL-21, IL-18, IL-7, and IL-12. The secreting enhancer, such as IL-15/IL-15sushi enhances CAR T or NK cell expansion and persistency. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion are stable and enhances CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

Figure 39B. Expression of CLL1-CD33b-IL15/IL-15sushi CAR T cells. Human peripheral blood buffy coat cells were activated 3 days with anti-human CD3 antibody. Cells were transduced with either control vector (left), CLL1-CD33b-IL15/IL15sushi CAR (right) lentiviral supernatant. After 4 days of incubation, cells were harvested and labeled for flow cytometry with goat anti-human F(Ab')₂ and mouse anti-human CD3 antibodies. CAR T cells are represented as green dots (circled).

Figure 39C CLL1-CD33b-IL15/IL15sushi CAR T cells specifically lyse the REH tumor cell line, which is synthetically expressing either CLL-1 or CD33 surface antigen, in co-culture assays. Each assay consisted of either REH-CLLxp (left graph) or REH-CD33xp target cells (right graph) co-cultured with control T cells or CLL1-CD33b-IL15/IL15sushi CAR T cells at 2:1 and 5:1 effector:target cell ratios. Co-culture experiments were performed for 24 hours and were directly analyzed by flow cytometry for anti-human CD3 and either CLL-1 or CD33.

Figure 40A. CLL1-CD33b-IL15/IL15sushi CAR T cells demonstrate strong anti-tumor effects *in vivo* against MOLM13 tumor cell line. NSG mice were sublethally irradiated and intravenously injected with 1.0×10^6 luciferase-expressing MOLM13 cells (Day 0) to induce measurable tumor formation. Starting 5 days after injection of tumor cells, mice were intravenously injected with a course of 15×10^6 either CLL1-CD33b-IL15/IL15sushi CAR T or vector control T cells. On days 4 (before T cell injection), 8 and 12, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. Dorsal view.

Figure 40B. CLL1-CD33b-IL15/IL15sushi CAR T cells demonstrate strong anti-tumor effects *in vivo* against MOLM13 tumor cell line. NSG mice were sublethally irradiated and intravenously injected with 1.0×10^6 luciferase-expressing MOLM13 cells (Day 0) to induce measurable tumor formation. Starting 5 days after injection of tumor cells, mice were intravenously injected with a course of 15×10^6 either CLL1-CD33b-IL15/IL15sushi CAR T or vector control T cells. On days 4 (before T cell injection), 8 and 12, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. Ventral view.

Figure 40C. (A) Peripheral blood was removed from mice from Figure 40A at time of sacrifice, labeled with mouse anti-human CD45, CD3 and CD33, and subjected to flow cytometry.

Transplanted human cells were gated by CD45, and analyzed for T cell (green dots) and MOLM13 cell (blue dots) populations. Results of two control mice are on the left, and the two mice treated with CLL1-CD33b-IL15/IL15sushi CAR T cells are on the right. (B) Plasma from the peripheral blood of each mouse was subjected to ELISA to quantify the amount of secreted human IL-15 fusion. Control mice, #1-2. Wells are in duplicate.

Figure 40D. CLL1-CD33b-IL15/IL15sushi NK cells express functional IL-15/IL-15 fusion. NK-92 cell line was transduced with lentiviral vector containing CLL1-CD33b-IL15/IL15sushi CAR. (A) Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype. (B) CLL1-CD33b-IL15/IL15sushi CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5×10^6 cells per mL, in 1 mL total volume. Cells were added to duplicate wells; one well of each pair contained IL-2 at 300 IU/mL, the other well did not. After 48 hours (Day 2), cells were counted (B), and the volume increased to yield a concentration of approximately 0.5×10^6 cells/mL. This process was repeated on Days 4, 6 and 8.

Figure 40E. Sorted CLL1-CD33b-IL15/IL15sushi NK cells and wild-type control NK-92 cells were cultured in separate wells for 72 hours. Supernatant was collected and subjected to ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained on a plate reader were compared to a standard curve (A) generated with human IL-15 to determine concentration of IL-15 in the supernatants (B).

Figure 41A. Expression of CD20cCD19b CAR T cells. Buffy coat cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (left), CD20cCD19b or CD20hCD19b CAR (right) lentiviral supernatant. After 3 days of incubation, cells were harvested and labeled for flow cytometry.

Figure 41B. CD20cCD19b and CD20hCD19b CAR T cells do not lyse K562 tumor cell line in co-culture assays. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 6 hours and were directly analyzed by flow cytometry for CD3 and CD45. Each assay consists of K562 target cells alone (right), control T cells (left) and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as blue dots. (N=2)

Figure 41C. cCAR T cells lyse CD19 synthetically-expressing K562 tumor cell line in co-culture assays. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 24 hours and were directly analyzed by flow cytometry for CD19 and CD3. Each assay consists of K562-CD19xp target cells (K562 expressing CD19, K-19) alone (right side), control T cells (left panels) and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as green dots.

Figure 41D. cCAR T cells lyse CD20 synthetically-expressing K562 tumor cell line, in co-culture assays. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 24 hours and were directly analyzed by flow cytometry for CD20 and CD3. Each assay consisted of K562-CD20xp target cells alone (right side), control T cells (left panels) and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as purple dots.

Figure 41E. cCAR T cells completely lyse CD19 -expressing REH tumor cell line in co-culture assays. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 24 hours and were directly analyzed by flow cytometry for CD19 and CD3. Each assay consisted of REH target cells alone (right side), control T cells (left panels) and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as orange dots.

Figure 41F. cCAR T cells completely lyse SP53 tumor cell line, which expresses both CD19 and CD20 antigens, in co-culture assay. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 24 hours and were directly analyzed by flow cytometry for CD19 and CD3. Each assay consisted of SP53 target cells alone (right side), control T cells (left panels) and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as turquoise dots. (N=2)

Figure 41G. A summary of the co-culture results.

Figure 42A. CD20hCD19b CAR T cells demonstrate anti-tumor effects *in vivo* against REH tumor cell line expressing CD19 antigen. NSG mice were sublethally irradiated and

intravenously injected with 1.0×10^6 luciferase-expressing REH cells (Day 0) to induce measurable tumor formation. Starting 6 days after injection of tumor cells, mice were intravenously injected with a course of 10×10^6 CD20hCD19b CAR T cells or vector control T cells. On days 5, 9 and 12, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. Ventral view.

Figure 42B. CD20hCD19b CAR T cells demonstrate anti-tumor effects *in vivo* against REH tumor cell line expressing CD19 antigen. NSG mice were sublethally irradiated and

intravenously injected with 1.0×10^6 luciferase-expressing REH cells (Day 0) to induce measurable tumor formation. Starting 6 days after injection of tumor cells, mice were intravenously injected with a course of 10×10^6 CD20hCD19b CAR T cells or vector control T cells. On days 5, 9 and 12, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. Ventral view.

Figure 43A. A schematic representation of cCAR-T with IL-15/IL15sushi enhancer construct.

The construct comprises a SFFV promoter driving the expression of multiple modular units of

CARs, and IL-15/IL-15sushi linked by P2A and T2A peptide respectively. Upon cleavage of the linker, the cCARs, CD20h-CD19b split and engage upon targets expressing CD20 and/or CD19 and a secreting enhancer fusion of IL-15/IL-15sushi. As a novel cCAR construct, the

activation domains of the construct may include, but is not limited to, 4-1BB or CD28 on the CD20h CAR segment and a CD28 or 4-1BB region on the CD19b CAR segment. The peptide

self cleavage peptides of the construct may include, but is not limited to, P2A, T2A, F2A and E2A. The secreting enhancer (s) of the construct may also include, but is not limited to, IL-

15/IL-15sush, IL-15, IL-21, IL-18, IL-7, and IL-12. The secreting enhancer, such as IL-15/IL-

15sushi enhances CAR T or NK T or NK cell expansion and persistency. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for

CAR T/NK cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion are stable and enhances CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects a by reprogramming body's immune system to fight cancers.

Figure 43B. Expression of CD20h-CD19b-IL15/IL15sushi CAR T cells. Human peripheral

blood buffy coat cells were activated 3 days with anti-human CD3 antibody. Cells were transduced with either control vector (left), or CD20h-CD19b-IL15/IL15sushi CAR (right)

lentiviral supernatant. After 4 days of incubation, cells were harvested and labeled for flow cytometry with goat anti-human F(Ab')₂ and mouse anti-human CD3 antibodies. CAR T cells are represented as green dots (circled).

Figure 43C. CD20h-CD19b-IL15/IL15sushi CAR T cells specifically lyse the U937 tumor cell line, which is synthetically expressing CD20 surface antigen (A), and REH tumor cells (B), which express the surface antigen CD19, in co-culture assays. Co-culture experiments were performed at an effector to target ratio of 2:1 or 5:1 for 48 hours and were directly analyzed by flow cytometry for anti-human CD20 and CD19, respectively.

Figure 44A. CD20h-CD19b-IL15/IL15sushi CAR T cells demonstrate strong anti-tumor effects *in vivo* against REH tumor cell line. NSG mice were sublethally irradiated and intravenously injected with 1.0×10^6 luciferase-expressing REH cells (Day 0) to induce measurable tumor formation. Starting 5 days after injection of tumor cells, mice were intravenously injected with a course of 15×10^6 either CD20h-CD19b-IL15/IL15sushi CAR T or vector control T cells. On days 4 (before T cell injection) 8 (72 hours after T cell injection), 12 and 16, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. Dorsal view.

Figure 44B. CD20h-CD19b-IL15/IL15sushi CAR T cells demonstrate strong anti-tumor effects *in vivo* against REH tumor cell line. NSG mice were sublethally irradiated and intravenously injected with 1.0×10^6 luciferase-expressing REH cells (Day 0) to induce measurable tumor formation. Starting 5 days after injection of tumor cells, mice were intravenously injected with a course of 15×10^6 either CD20h-CD19b-IL15/IL15sushi CAR T or vector control T cells. On days 4 (before T cell injection) 8 (72 hours after T cell injection), 12 and 16, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. Ventral view.

Figure 44C. (A) Peripheral blood was removed from mice from Figure 3 at time of sacrifice, labeled with mouse anti-human CD45, CD3 and CD19, and subjected to flow cytometry.

Transplanted human cells were gated by CD45, and analyzed for T cell (green dots) and REH cell (blue dots) populations. Results of two control mice are on the left, and the two mice treated with CD20h-CD19b-IL15/IL15sushi CAR T cells are on the right. (B) Plasma from the peripheral blood of each mouse was subjected to ELISA to quantify the amount of secreted IL-15. Control mice, #1-2.

Figure 44D. CD20h-CD19b-IL15/IL15sushi NK cells express functional IL15. NK-92 cell line was transduced with lentiviral vector containing CD20h-CD19b-IL15/IL15sushi-IL15/IL15sushi

CAR. (A) Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype. (B) CD20h-CD19b-IL15/IL15sushi-IL15/IL15sushi CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5×10^6 cells per mL, in 1 mL total volume. Cells were added to duplicate wells; one well of each pair contained IL-2 at 300 IU/mL, the other well did not. After 48 hours (Day 2), cells were counted (B), and the volume increased to yield a concentration of approximately 0.5×10^6 cells/mL. This process was repeated on Days 4, 6 and 8.

Figure 44E. Sorted CD20h-CD19b-IL15/IL15sushi NK cells and wild-type control NK-92 cells were cultured in separate wells for 72 hours. Supernatant was collected and subjected to ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained on a plate reader were compared to a standard curve (A) generated with human IL-15 to determine concentration of IL-15 in the supernatants (B).

Figure 45A. Expression was measured by FACS against control T-cells. Upper is the organization of CD19b-IL-15/IL-15sushi CAR. CD19b-IL-15/IL-15sushi CAR T-cells are created by the viral transduction of patient or donor T-cells with lentiviruses expressing CD19b-IL-15/IL-15sushi CAR and the transduced T cells are able to secrete IL-15/IL-15sushi fusion protein.. FACS analysis shows that CD19b-IL-15/IL-15sushi CAR is able to be expressed on 35% of the T cells, (bottom) furthermore, the secreting IL-15/IL-15sushi fusion provides additional stimulation, proliferation, and potency enhancement to the CAR T cells or NK cells when compared to a standard CAR cell.

FIGURE 45B: CD19b-IL15/IL15sushi CAR T-cells potently lyse CD19+ Sp53 cells.

FIGURE 45C: CD19b-IL15/IL-15sushi CAR T-cells potently lyse CD19+ Sp53 cells (with comparison to CD19b single CAR T).

Figure. 46A – CD19 based CARs deplete Reh cells in vivo and IL-15/IL-15sushi conjugates augment anti-tumor response. Mice were injected with Reh tumor cells (0.5×10^6 cells/mouse) expressing luciferase on Day 1. On Day 3 IVIS was conducted to assay the appearance of circulating Reh cells. On Day 4, control T-cells, CD19b CAR, and CD19b-IL15/IL-15sushi CAR T-cells were injected ($\sim 7.5 \times 10^6$ total cells/mouse) and on day 6 through 22, IVIS imaging was conducted to assay semi-quantitative assessment of tumor burden and subsequent tumor depletion and control of cell growth by T-cells. Here, both CAR T treatments demonstrated similar efficacy, with the IL-15 secreting CAR demonstrating comparable or better control of the

Reh tumor growth when compared to standard CART19 cells.

Figure 46B - Comparison CD19b-CAR-T vs CD19b-IL-15/IL15sushi CAR-T against REH cells over long term. (TOP) Similar experimental scheme with identical IVIS methodology to

above, however mice were followed until signs of tumor relapse were seen. Here, after day 30,

we observed that aggressive Reh tumor relapse began to occur in standard CART19 treated mice.

Clusters of tumor (indicated by red regions on the IVIS imaged mice) are seen in most CART19

mice, with a single CD19b-IL-15/IL-15sushi CART treated mice also showing tumor growth by

day 22. However, after day 30, all CART19 mice show signs of severe tumor relapse, while

CD19b-IL-15/IL-15sushi CART treated mice show no sign of tumor. Even the relapsed mouse

on day 22 was absolved of its tumor by day 32, signifying that CD19b-IL-15/IL-15sushi CART cells were still in effective circulation.

Figure 46C. Line graph summarizing IVIS trend values estimating tumor growth over time for

each treatment cohort. Past day 30, the tumor burden for the standard CD19b CAR (CART19)

treated mice rises precipitously resulting in highly significant increases in tumor burden

compared to the CD19b-IL-15/IL-15sushi CART treatment group which remained largely tumor free. Values are displayed for both views of the mice (dorsal image acquisition views).

Figure 46D – Overall summary of mice blood data-summarized persistence of T cells in mice.

The overall persistence of T cells in mouse blood from the model in Fig. 3A-1 was

assayed at survival endpoints and screened by flow cytometry using CD3 antibody for bulk T

cell populations. To further dissect the persistency results of the CD19b-IL-15/IL-15sushi

armored CAR, the collection of mouse blood is necessary to reveal the presence of durability of

the engrafted human cells. Overall, we find by flow cytometry analysis that there is a higher

average count of T cells in the IL-15/il-15sushi secreting CAR cohorts when compared to the

standard CART19 groups. Control group T cells remain at baseline as expected due to minimal

stimulation from circulating in *vivo* tumor.

Figure. 46E – Mice blood data (individual). Representative mice from Fig. 3A-1 was assayed

by flow cytometry at survival endpoints to screen for remaining tumor and T cell populations,

revealed by CD19+ and CD3+ expressions respectively. These flow cytometry plots further

analyze the conditions of each mouse at their survival endpoints revealing that significant tumor

populations and diminished T cell counts are characteristic of control and most CART19 mice.

Significant counts of T cells and few or absence tumor cells are characteristic of the CD19b-IL-

15/IL-15sushi CAR cohort mice.

Figure 47A. Expression of CD269-A7D-CD38 CAR T cells. Buffy coat cells were activated 3 days with anti-CD3 antibody. Cells were transduced with either control vector (left), CD269-A7D-CD38a, CD269-A7D-CD38b, or CD269-A7D-CD38c CAR (right) lentiviral supernatant.

5 After 3 days of incubation, cells were harvested and labeled for flow cytometry.

Figure 47B. CD269-A7D-CD38 CAR T cells specifically lyse the REH tumor cell line, which expresses CD38 surface antigen, in co-culture assays. Co-culture experiments were performed at an effector to target ratio of 2:1 (top row) or 5:1 (bottom row) for 24 hours and were directly analyzed by flow cytometry for CD38 and CD3. Each assay consists of REH target cells
10 incubated with control T cells (left panels), CD269-A7D-CD38a (center left panels) or CD269-A7D-CD38a CAR T cells (center-right panels), or cells alone (far right). REH cells are represented as blue dots.

Figure 47C. CD269-A7D-CD38 CAR T cells specifically lyse the K562 tumor cell line, which is synthetically expressing CD269 (BCMA) surface antigen, in co-culture

15 assays. Co-culture experiments were performed at an effector to target ratio of 2:1 (top row) or 5:1 (bottom row) for 24 hours and were directly analyzed by flow cytometry for CD269 and CD3. Each assay consists of K562-BCMA target cells incubated with control T cells (left panels), CD269-A7D-CD38b (center left panels) or CD269-A7D-CD38a CAR T cells (center-right panels), or cells alone (far right). K-BCMA cells are represented as green dots.

20 **Figure 48A.** A schematic representation of cCAR-T with IL-15/IL15sushi enhancer construct.

The construct comprises a SFFV promoter driving the expression of multiple modular units of CARs, and IL-15/IL-15sushi linked by P2A and T2A peptide respectively. Upon cleavage of the linker, the cCARs split and engage upon targets expressing BCMA and/or CD38 and a secreting enhancer fusion of IL-15/IL-15sushi. As a novel cCAR construct, the activation
25 domains of the construct may include, but is not limited to, 4-1BB or CD28 on the BCMA CAR segment and a CD28 or 4-1BB region on the CD38 CAR segment. The peptide self cleavage peptides of the construct may include, but is not limited to, P2A, T2A, F2A and E2A. The secreting enhancer (s) of the construct may also include, but is not limited to, IL-15/IL-15sush, IL-15, IL-21, IL-18, IL-7, and IL-12. The secreting enhancer, such as IL-15/IL-15sushi
30 enhances CAR T or NK T or NK cell expansion and persistency. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR

T/NK cells or NK T cells and their neighbor tumor immune response cells. . The soluble IL-15/IL-15sushi fusion are stable and enhances CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity . The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects a by reprogramming body's immune system to fight
5 cancers.

Figure 48B. Expression of CD269-A7D-CD38a-IL15/IL15sushi CAR T cells. Human peripheral blood buffy coat cells were activated 3 days with anti-human CD3 antibody. Cells were transduced with either control vector (left), CD269-A7D-CD38a-IL15/IL15sushi CAR (right) lentiviral supernatant. After 4 days of incubation, cells were harvested and labeled for flow
10 cytometry with goat anti-human F(Ab')₂ and mouse anti-human CD3 antibodies. CAR T cells are represented as green dots (circled).

Figure 48C. CD269-A7D-CD38a-IL15/IL15sushi CAR T cells lyse CD38+ T cells. Human peripheral blood buffy coat cells were activated 3 days with anti-human CD3 antibody. Cells were transduced with either control vector (left), CD269-A7D-CD38a-IL15/IL15sushi CAR
15 (right) lentiviral supernatant. After 6 days (two left graphs) and 12 days (two right graphs) of incubation, cells were harvested and labeled for flow cytometry with mouse anti-human CD3 and CD38 antibodies. CD38+ T cells are represented as blue dots (circled).

Figure 48D. CD269-A7D-CD38a-IL15/IL15sushi CAR T cells specifically lyse the K562 tumor
20 cell line, which is synthetically expressing BCMA (CD269) surface antigen, and wild-type REH cells, which naturally express CD38 antigen, in co-culture assays. Each assay consisted of either K562-BCMAxp (left graph) or REH target cells (right graph) co-cultured with control T cells or CD269-A7D-CD38a-IL15/IL15sushi CAR T cells at 2:1 and 5:1 effector:target cell ratios. Co-culture experiments were performed for 24 and 48 hours and were directly analyzed by flow
25 cytometry for anti-human CD3 and either CD269 or CD38.

Figure 48E. CD269-A7D-CD38a-IL15/IL15sushi NK cells express functional IL15. NK-92 cell line was transduced with lentiviral vector containing CD269-A7D-CD38a-IL15/IL15sushi CAR.
(A) Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype. NK CAR cells are represented by blue dots (circled). (B) CD269-A7D-CD38a-IL15/IL15sushi
30 CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5 x 10⁶ cells per mL, in 1 mL total volume. Cells were added to duplicate wells; one well of each pair

contained IL-2 at 300 IU/mL, the other well did not. After 48 hours (Day 2), cells were counted (B), and the volume increased to yield a concentration of approximately 0.5×10^6 cells/mL. This process was repeated on Days 4, 6, and 8.

Figure 48F. Sorted CD269-A7D-CD38a-IL15/IL15sushi NK cells and wild-type control NK-92 cells were cultured in separate wells for 72 hours. Supernatant was collected and subjected to ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained on a plate reader were compared to a standard curve (A) generated with human IL-15 to determine concentration of IL-15 in the supernatants (B).

Figure 49A. Expression of CD123b-CD33b-IL15/IL15sushi and CD123b-CLL1-IL15/IL15sushi CAR T cells. Human peripheral blood buffy coat cells were activated 3 days with anti-human CD3 antibody. Cells were transduced with either control vector (left), CD123b-CD33b-IL15/IL15sushi (center) or CD123b-CLL1-IL15/IL15sushi CAR (right) lentiviral supernatant. After 4 days of incubation, cells were harvested and labeled for flow cytometry with goat anti-human F(Ab')₂ and mouse anti-human CD3 antibodies. CAR T cells are represented as green dots (circled).

Figure 49B. CD123b-CD33b-IL15/IL15sushi and CD123b-CLL1-IL15/IL15sushi NK cells express functional IL15. NK-92 cell line was transduced with lentiviral vector containing CD123b-CD33b-IL15/IL15sushi or CD123b-CLL1-IL15/IL15sushi CAR. (A) Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype. NK CAR cells are represented by blue dots (circled). (B) CD123b-CD33b-IL15/IL15sushi and CD123b-CLL1-IL15/IL15sushi CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5×10^6 cells per mL, in 1 mL total volume. Cells were added to duplicate wells; one well of each pair contained IL-2 at 300 IU/mL, the other well did not. After 48 hours (Day 2), cells were counted (B), and the volume increased to yield a concentration of approximately 0.5×10^6 cells/mL. This process was repeated on Days 4, 6, and 8.

Figure 49C. Sorted CD123b-CD33b-IL15/IL15sushi and CD123b-CLL1-IL15/IL15sushi NK cells and wild-type control NK-92 cells were cultured in separate wells for 72 hours. Supernatant was collected and subjected to ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained on a plate reader were compared to a standard curve (A) generated with human IL-15 to determine concentration of IL-15 in the supernatants (B).

Figure 50A. A schematic representation of anti-BCMA CAR with IL-15/IL15sushi enhancer construct (BCMA-IL-15/IL15sushi, also called CD269-A7D-IL-15/IL-15sushi). The construct comprises a SFFV promoter driving the expression of anti-BCMA CAR, and IL-15/IL-15sushi linked by P2A or T2A peptide respectively. Upon cleavage of the linker, the CAR split and engage upon targets expressing BCMA and a secreting enhancer fusion of IL-15/IL-15sushi. As a novel CAR construct, the activation domains of the construct may include, but is not limited to, CD28 or 4-1BB on the anti-BCMA CAR segment. The peptide self cleavage peptides of the construct may include, but is not limited to, P2A, T2A, F2A and E2A. The secreting enhancer (s) of the construct may also include, but is not limited to, IL-15/IL-15sush, IL-15, IL-21, IL-18, IL-7 and IL-12. The secreting enhancer, such as IL-15/IL-15sushi enhances CAR T or NK cell expansion and persistency. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. . The soluble IL-15/IL-15sushi fusion are stable and enhances CAR T/NK or NK T cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects a by reprogramming body's immune system particularly NK cells to fight cancers.

Figure 50B. Steps for generation and preparation of irradiated genetically modified K562 cells as feeder cells for primary NK cell expansion.

Figure 50C. Steps for generation and expansion of CAR-transduced natural killer (NK) cells from umbilical cord blood by co-culture with irradiated genetically modified K562 cells (feeder cells)

Figure 51A. BCMA-IL15/IL15sushi-CAR NK cells demonstrate anti-leukemic effects in vivo mouse model. NSG mice were sublethally irradiated and intravenously injected the following day with luciferase-expressing MM1S multiple myeloma cells to induce measurable tumor formation. On Day 4, the mice were intravenously injected with 10×10^6 Control NK cells or BCMA-IL15/IL15sushi-CAR expressed NK cells. On Days 3, 6, 8 and 10, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging.

Figure 51B. Comparison of total flux values (photons /sec) between control and BCMA-IL15/IL15sushi-CAR expressed NK cells treated mice over time. The data are presented as a mean + S.D. *, $P < 0.05$ as compared to control NK-cells at indicated days.

Figure 51C. Survival comparison of mice injected with MM1S multiple myeloma cells between

control mice and mice treated with BCMA-IL15/IL15sushi-CAR NK cells over time. A survival curve was generated to show the survival of mice over time, up to Day 80, with Log-rank (Mantel-Cox) Test $p = 0.0069$.

Figure 52A. Evaluation of persistence of infused BCMA-A7D-IL15/IL15sushi CAR transduced NK cells in xenograft mouse model on Day 25 (**D**) and Day 60 (**E**). On Day 25 (21 days after control NK or CAR NK cells infused mice) and Day 60 (58 days after mice were infused with control NK or CAR NK cells), peripheral blood was collected from individual mice and cells were labeled using human CD56-and human CD45 antibodies to detect the presence of infused control- and/or CAR- NK cells. The persistence of control NK cells or BCMA-IL15/IL-15sushi CAR transduced NK cells in collected peripheral blood was determined by flow cytometry analysis. Left panels show the negative controls, in which an NK cells were uninfused. The middle panels show the group of mice infused with control NK cells. Right panels show the group of mice infused with BCMA-IL15/IL15sushi-CAR transduced NK cells.

Figure 52B. Evaluation of persistence of infused BCMA-A7D-IL15/IL15sushi CAR transduced NK cells in xenograft mouse model on Day 60.

Figure 53. Schematic diagram to elucidate the construct of a CAR co-expressing a secreting IL-15/IL-15sushi fusion protein and its expression in T or NK cells. A) a combination of CAR (third or two generation) and IL15/sushi domain of the IL15 alpha receptor, is assembled on an expression vector, and their expression is driven by the SFFV promoter. CAR with IL-15/sushi is linked with the P2A self-cleaving sequence. The IL-15/sushi portion is composed of IL-2 signal peptide fused to IL-15 and linked to sushi domain via a 26-amino acid poly-proline linker. B) CAR and IL-15/sushi are present on the T or NK cells. The peptide self cleavage peptides of the construct may include, but is not limited to, P2A, T2A, F2A and E2A. The secreting enhancer (s) of the construct may also include, but is not limited to, IL-15/IL-15sushi, IL-15, IL-21, IL-18, IL-7 and IL-12. The secreting enhancer, such as IL-15/IL-15sushi enhances CAR T or NK cell expansion and persistency. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. . The soluble IL-15/IL-15sushi fusion are stable and enhances CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte

proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

Figure 54. A schematic showing a CAR equipped with IL-15/IL-15sushi anchor. A) the construct consists a SFFV promoter driving the expression of a CAR and an IL-15/IL-15sushi anchor (also called anchor) linked by a P2A peptide. Upon cleavage of this P2A peptide, IL-15/IL-15 anchor CAR splits to a CAR and an IL-15/IL-15sushi anchor. The IL-15/IL-15sushi portion of anchor is composed of IL-2 signal peptide fused to IL-15 and linked to sushi domain of IL-15 alpha receptor via a 26-amino acid poly-proline linker. Both CAR and anchor comprise a hinge (H) region, a transmembrane domain (TM). CAR also has scFv, costimulatory domain (including, but not limited to CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain while anchor does not bear these components. B) IL-15/IL-15sushi is anchored on the surface of T or NK cells.

Figure 55. A schematic showing a CAR enhancer construct. The construct consists a SFFV promoter driving the expression of a CAR and an enhancer, 4-1BBL (CD137L) linked by a P2A peptide. Upon cleavage of this P2A peptide, A CAR construct with 4-1BBL splits to a CAR and the full length of 4-1BBL protein. A CAR comprises a leader sequence and scFv, a hinge (H) region, a transmembrane domain (TM). CAR also has costimulatory domain (including, but not limited to, CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain while 4-1BBL does not bear these components. 4-1BBL provides a synergistic effect of T cell activation or anti-tumor activity with CD28 or 4-1BB (but not limited to)

Figure 56. A schematic showing a CAR enhancer construct. The construct consists a SFFV promoter driving the expression of a CAR and an enhancer, IL-15 linked by a P2A peptide. Upon cleavage of this P2A peptide, A CAR construct with IL-15 splits to a CAR and the full length of IL-15 protein. A CAR comprises a leader sequence and scFv, a hinge (H) region, a transmembrane domain (TM). CAR also has a costimulatory domain (including, but not limited to, CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain while IL-15 does not bear these components. IL-15 provides a synergistic effect of T cell activation or expansion or anti-tumor activity with CD28 or 4-1BB. The IL-15 signal peptide in the IL-15 is replaced with IL-2 signal peptide (leader sequence), a strong signal peptide to provide a high efficiency of IL-15 secretion.

Figure 57. A schematic showing a CAR construct with multiple enhancers (CAR super). The construct consists a SFFV promoter driving the expression of a CAR and enhancers, 4-1BBL

(CD137L) and IL-15/IL-15sushi linked by a P2A and T2A peptide, respectively. Upon cleavage of this P2A and T2A peptides, A CAR construct with 4-1BBL and IL-15/IL-15sushi splits to a CAR and the full length of 4-1BBL protein, and secreting IL-15/IL-15sushi . A CAR comprises a leader sequence and scFv, a hinge (H) region, a transmembrane domain (TM). CAR also has costimulatory domain (including, but not limited to, CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain. 4-1BBL ligand provides a synergistic effect of T or NK cell activation or anti-tumor activity with CD28 or 4-1BB (but not limited to). The peptide self cleavage peptides of the construct may include, but is not limited to, P2A, T2A, F2A and E2A. The secreting enhancer (s) of the construct may also include, but is not limited to, IL-15/IL-15sush, IL-15, IL-21, IL-18, IL-7, and IL-12. The secreting enhancer, such as IL-15/IL-15sushi enhances CAR T or NK cell expansion and persistency. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK or NK T cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion are stable and enhances CAR T/NK or NK T cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity . The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

Figure 58. Generation of CD123b-Super-1-CAR expressed human NK cells derived from human cord blood. Flow cytometry analysis showed the expression levels of CD123b-Super 1CAR on CD56 positive cells (circled in pink) in cord blood cells after transduction CD123b Super-1 CAR viruses in cord blood cells.

Figure 59. Generation of BCMA-CD38a-IL15/IL15sushi cCAR (also called CD269-A7D-CD38a-IL15/IL15sushi) CAR. BCMA-CD38a-IL15/IL15sushi cCAR was expressed in human NK cells derived from human cord blood. Flow cytometry analysis showed the expression levels of BCMA-CD38a-IL15/IL15sushi- cCAR on CD56 positive cells (circled in pink) in cord blood cells after transduction BCMA-CD38a-IL-15/IL-15sushi cCAR viruses in cord blood cells.

Figure 60A. A schematic representation of a super1 CAR construct. Links by P2A and T2A schematic to generate a super1 CAR showing a CAR, GD2 CAR equipped with 4-1BBL and IL-15/IL-15sushi in a single construct. The construct consists of a SFFV promoter driving the expression of three segments, CAR, 4-1BBL and IL-15/IL-15sushi. Upon cleavage of the linkers (P2A and T2A), the CAR (GD2 CAR), 4-1BBL and IL-15/IL-15sushi split and engage upon a target (s). CAR has scFv, hinge region, transmembrane domain, costimulatory domain

(including, but not limited to, CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain. 4-1BBL or IL-15/IL-sushi or both provides a synergistic effect of T or NK cell activation and persistency or anti-tumor activity with CD28 or 4-1BB.

5 **Figure 60B. GD2-Super1-CAR-T cells virtually eliminate Y79 cells in mouse liver**

(A) Flow cytometry analysis shows persistence of Y79 tumor (Blue dots) in the livers of mice treated with different forms of anti-GD2 CAR T cells. Three days after Y79 cells (1×10^6 cells) were injected mice via tail vein, CAR T-cells (10×10^6 cells) were infused into mice by I.V. injection. At day 30 after Y79 tumor injection, mice were euthanized and livers were
10 homogenized to evaluate CAR T efficacy. Homogenized liver cells were labeled with mouse anti-human CD3 and CD56 antibodies to detect human T cells and Y79 tumor cells, respectively. A representation of a mouse given control T cells is shown on the left; mouse treated with GD2CAR (left center), GD2-4-1BBL CAR (right center), and GD2-Super1 CAR (right) T cells. Elimination of tumor cells was associated with high labels of T-cells. GD2-4-BBL CAR is a
15 GD2 CAR co-expressing 4-1BBL ligand.

(B) Graph indicating percent killing activity against Y79 cells by each CAR treated mice compared to control mice ($n=2$). From these data, especially, only GD2 Super1 CAR T were able to virtually eliminate Y79 cells in liver.

20 **Figure 60C. GD2-Super1-CAR T cells exhibit greater persistence in mouse spleen**

(A) Flow cytometry analysis shows persistence of CAR T cells (circled) in the livers of mice treated with different forms of anti-GD2 CAR T cells. Three days after Y79 cells (1×10^6 cells) were injected mice via tail vein, CAR T-cells (10×10^6 cells) were infused into mice by I.V. injection. At day 30 after Y79 tumor injection, mice were euthanized and spleens were
25 homogenized to evaluate CAR T efficacy. Homogenized spleen cells were labeled with mouse anti-human CD3 and CD45 antibodies to detect human T cells. A representation of a mouse given control T cells is shown on the left; mouse treated with GD2CAR (left center), GD2-4-1BBL CAR (right center), and GD2-super1 CAR (right) T cells.

(B) Graph indicating fold-increase of CAR T cells in treated mice compared to control T mice
30 ($n=2$). From these data, especially, GD2-Super CAR T cells were well expanded compared to control T-cells in total mouse spleen cells.

Figure 60D. Persistence of CAR T cells in mouse blood

(A) Flow cytometry analysis shows persistence of CAR T cells (circled) in the whole blood of mouse treated with different forms of anti-GD2 CAR T cells. Three days after Y79 cells

5 (1x10⁶ cells) were injected mice via tail vein, CAR T-cells (10x10⁶ cells) were infused into mice by I.V. injection. At day 30 after Y79 tumor injection, mice were euthanized and whole blood was collected to evaluate CAR T persistence. Whole blood cells were labeled with mouse anti-human CD3 and CD45 antibodies, to detect human T cells. A representation of a mouse given control T cells is shown on the left; mice treated with GD2CAR (left center), GD2-4-1BBL CAR
10 (right center), and GD2-Super1 CAR (right) T cells.

Figure 60E. Bar graph representing the percent persistence of human T cells in whole blood samples, relative to the number of total cells analyzed by flow cytometry (n=2 each)

15

DETAILED DESCRIPTION

The disclosure provides chimeric antigen receptor (CAR) compositions, methods and
20 making thereof, and methods of using the CAR compositions.

COMPOSITIONS**Chimeric Antigen Receptor Polypeptides**

In one embodiment, the disclosure provides a chimeric antigen receptor (CAR)
25 polypeptide having a signal peptide, an antigen recognition domain, a hinge region, a transmembrane domain, at least one co-stimulatory domain, and a signaling domain.

As used herein, the terms “peptide,” “polypeptide,” and “protein” are used interchangeably, and refer to a compound having amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is
30 placed on the maximum number of amino acids that can include a protein's or peptide's sequence. Polypeptides include any peptide or protein having two or more amino acids joined to

each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides, and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially

5 homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

A "signal peptide" includes a peptide sequence that directs the transport and localization of the peptide and any attached polypeptide within a cell, e.g. to a certain cell organelle (such as
10 the endoplasmic reticulum) and/or the cell surface.

The signal peptide is a peptide of any secreted or transmembrane protein that directs the transport of the polypeptide of the disclosure to the cell membrane and cell surface, and provides correct localization of the polypeptide of the present disclosure. In particular, the signal peptide of the present disclosure directs the polypeptide of the present disclosure to the cellular
15 membrane, wherein the extracellular portion of the polypeptide is displayed on the cell surface, the transmembrane portion spans the plasma membrane, and the active domain is in the cytoplasmic portion, or interior of the cell.

In one embodiment, the signal peptide is cleaved after passage through the endoplasmic reticulum (ER), i.e. is a cleavable signal peptide. In an embodiment, the signal peptide is human
20 protein of type I, II, III, or IV. In an embodiment, the signal peptide includes an immunoglobulin heavy chain signal peptide.

The "antigen recognition domain" includes a polypeptide that is selective for an antigen, receptor, peptide ligand, or protein ligand of the target; or a polypeptide of the target.

The target specific antigen recognition domain preferably includes an antigen binding
25 domain derived from an antibody against an antigen of the target, or a peptide binding an antigen of the target, or a peptide or protein binding an antibody that binds an antigen of the target, or a peptide or protein ligand (including but not limited to a growth factor, a cytokine, or a hormone) binding a receptor on the target, or a domain derived from a receptor (including but not limited to a growth factor receptor, a cytokine receptor or a hormone receptor) binding a peptide or protein
30 ligand on the target. The target includes GD2 and GD3. In another embodiment, the target includes any portion of GD2 and GD3. In another embodiment, the target is gangliosides GD2

with its structure, GD2 = bDGalpNAc(1-4)[aNeu5Ac(2-8)aNeu5Ac(2-3)]bDGalp(1-4)bDGlc(1-1)Cer. In another embodiment, the target is the gangliosides GD3 with its structure, GD3 = aNeu5Ac(2-8)aNeu5Ac(2-3)bDGalp(1-4)bDGlc(1-1)Cer.

In one embodiment, the antigen recognition domain includes the binding portion or
5 variable region of a monoclonal or polyclonal antibody directed against (selective for) the target.

In one embodiment, the antigen recognition domain includes antigen-binding fragment (Fab). In another embodiment, the antigen recognition domain includes a single-chain variable fragment (scFv). scFv is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide.

10 In another embodiment, the antigen recognition domain includes Camelid single domain antibody, or portions thereof. In one embodiment, Camelid single-domain antibodies include heavy-chain antibodies found in camelids, or VHH antibody. A VHH antibody of camelid (for example camel, dromedary, llama, and alpaca) refers to a variable fragment of a camelid single-chain antibody (See Nguyen et al, 2001; Muyldermans, 2001), and also includes an isolated
15 VHH antibody of camelid, a recombinant VHH antibody of camelid, or a synthetic VHH antibody of camelid.

In another embodiment, the antigen recognition domain includes ligands that engage their cognate receptor. In another embodiment, the antigen recognition domain is humanized.

It is understood that the antigen recognition domain may include some variability within
20 its sequence and still be selective for the targets disclosed herein. Therefore, it is contemplated that the polypeptide of the antigen recognition domain may be at least 95%, at least 90%, at least 80%, or at least 70% identical to the antigen recognition domain polypeptide disclosed herein and still be selective for the targets described herein and be within the scope of the disclosure.

In another embodiment, the antigen recognition domain is selective for gangliosides GD2
25 and gangliosides GD3.

The hinge region is a sequence positioned between for example, including, but not limited to, the chimeric antigen receptor, and at least one co-stimulatory domain and a signaling domain. The hinge sequence may be obtained including, for example, from any suitable sequence from any genus, including human or a part thereof. Such hinge regions are known in
30 the art. In one embodiment, the hinge region includes the hinge region of a human protein including CD-8 alpha, CD28, 4-1BB, OX40, CD3-zeta, T cell receptor α or β chain, a CD3 zeta

chain, CD28, CD3 ϵ , CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, ICOS, CD154, functional derivatives thereof, and combinations thereof.

In one embodiment the hinge region includes the CD8a hinge region.

In some embodiments, the hinge region includes one selected from, but is not limited to, immunoglobulin (e.g. IgG1, IgG2, IgG3, IgG4, and IgD).

The transmembrane domain includes a hydrophobic polypeptide that spans the cellular membrane. In particular, the transmembrane domain spans from one side of a cell membrane (extracellular) through to the other side of the cell membrane (intracellular or cytoplasmic).

The transmembrane domain may be in the form of an alpha helix or a beta barrel, or combinations thereof. The transmembrane domain may include a polytopic protein, which has many transmembrane segments, each alpha-helical, beta sheets, or combinations thereof.

In one embodiment, the transmembrane domain that naturally is associated with one of the domains in the CAR is used. In another embodiment, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

For example, a transmembrane domain includes a transmembrane domain of a T-cell receptor α or β chain, a CD3 zeta chain, CD28, CD3 ϵ , CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, ICOS, CD154, functional derivatives thereof, and combinations thereof.

The artificially designed transmembrane domain is a polypeptide mainly comprising hydrophobic residues such as leucine and valine. In one embodiment, a triplet of phenylalanine, tryptophan and valine is found at each end of the synthetic transmembrane domain.

In one embodiment, the transmembrane domain is the CD8 transmembrane domain. In another embodiment, the transmembrane domain is the CD28 transmembrane domain. Such transmembrane domains are known in the art.

The signaling domain and co-stimulatory domain include polypeptides that provide activation of an immune cell to stimulate or activate at least some aspect of the immune cell signaling pathway.

In an embodiment, the signaling domain includes the polypeptide of a functional signaling domain of CD3 zeta, common FcR gamma (FCER1G), Fc gamma R1a, FcR beta (Fc

Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD79a, CD79b, DNAX-activating protein 10 (DAP10), DNAX-activating protein 12 (DAP12), active fragments thereof, functional derivatives thereof, and combinations thereof. Such signaling domains are known in the art.

In an embodiment, the CAR polypeptide further includes one or more co-stimulatory
5 domains. In an embodiment, the co-stimulatory domain is a functional signaling domain (s) selected from at least a protein including, but not limited to, IL-15 receptor alpha; IL-15 receptor alpha cytoplasmic domain; B7-1/CD80; CD28; 4-1BB, 4-1BBL, B7-2/CD86; CTLA-4; B7-H1/PD-L1; ICOS; B7-H2; PD-1; B7-H3; PD-L2; B7-H4; PDCD6; BTLA; 4-1BB/TNFRSF9/CD137; CD40 Ligand/TNFSF5; 4-1BB Ligand/TNFSF9; GITR/TNFRSF18;
10 BAFF/BLyS/TNFSF13B; GITR Ligand/TNFSF18; BAFF R/TNFRSF13C; HVEM/TNFRSF14; CD27/TNFRSF7; LIGHT/TNFSF14; CD27 Ligand/TNFSF7; OX40/TNFRSF4; CD30/TNFRSF8; OX40 Ligand/TNFSF4; CD30 Ligand/TNFSF8; TACI/TNFRSF13B; CD40/TNFRSF5; 2B4/CD244/SLAMF4; CD84/SLAMF5; BLAME/SLAMF8; CD229/SLAMF3; CD2, CD27, CRACC/SLAMF7; CD2F-10/SLAMF9; NTB-A/SLAMF6;
15 CD48/SLAMF2; SLAM/CD150; CD58/LFA-3; Ikaros; CD53; Integrin alpha 4/CD49d; CD82/Kai-1; Integrin alpha 4 beta 1; CD90/Thy1; Integrin alpha 4 beta 7/LPAM-1; CD96; LAG-3; CD160; LMIR1/CD300A; CRTAM; TCL1A; DAP12; TIM-1/KIM-1/HAVCR; Dectin-1/CLEC7A; TIM-4; DPPIV/CD26; TSLP; EphB6; TSLP R; and HLA-DR.

The present disclosure further provides a polynucleotide encoding the chimeric antigen
20 receptor polypeptide described above. The polynucleotide encoding the CAR is easily prepared from an amino acid sequence of the specified CAR by any conventional method. A base sequence encoding an amino acid sequence can be obtained from the aforementioned NCBI RefSeq IDs or accession numbers of GenBank for an amino acid sequence of each domain, and the nucleic acid of the present disclosure can be prepared using a standard molecular biological
25 and/or chemical procedure. For example, based on the base sequence, a polynucleotide can be synthesized, and the polynucleotide of the present disclosure can be prepared by combining DNA fragments which are obtained from a cDNA library using a polymerase chain reaction (PCR).

In one embodiment, the polynucleotide disclosed herein is part of a gene, or an
30 expression or cloning cassette.

The term “polynucleotide” as used herein is defined as a chain of nucleotides.

Polynucleotide includes DNA and RNA. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric “nucleotides.” The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and polymerase chain reaction (PCR), and the like, and by synthetic means.

Polynucleotide vector

The polynucleotide described above can be cloned into a vector. A “vector” is a composition of matter which includes an isolated polynucleotide and which can be used to deliver the isolated polynucleotide to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, phagemid, cosmid, and viruses. Viruses include phages, phage derivatives. Thus, the term “vector” includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like.

In one embodiment, vectors include cloning vectors, expression vectors, replication vectors, probe generation vectors, integration vectors, and sequencing vectors.

In an embodiment, the vector is a viral vector. In an embodiment, the viral vector is a retroviral vector or a lentiviral vector. In an embodiment, the engineered cell is virally transduced to express the polynucleotide sequence.

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject

either in vivo or ex vivo. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

Viral vector technology is well known in the art and is described, for example, in
5 Sambrook et al, (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno- associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction
10 endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

Expression of chimeric antigen receptor polynucleotide may be achieved using, for example, expression vectors including, but not limited to, at least one of a SFFV (spleen focus-forming virus) or human elongation factor 1 α (EF) promoter, CAG (chicken beta-actin
15 promoter with CMV enhancer) promoter human elongation factor 1 α (EF) promoter. Examples of less-strong/ lower-expressing promoters utilized may include, but is not limited to, the simian virus 40 (SV40) early promoter, cytomegalovirus (CMV) immediate-early promoter, Ubiquitin C (UBC) promoter, and the phosphoglycerate kinase 1 (PGK) promoter, or a part thereof. Inducible expression of chimeric antigen receptor may be achieved using, for example, a
20 tetracycline responsive promoter, including, but not limited to, TRE3GV (Tet-response element, including all generations and preferably, the 3rd generation), inducible promoter (Clontech Laboratories, Mountain View, CA) or a part or a combination thereof.

One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable
25 of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable promoter is Elongation Growth Factor - 1 α (EF- 1 α). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an
30 avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter,

the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the disclosure should not be limited to the use of constitutive promoters, inducible promoters are also contemplated as part of the disclosure. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is
5 operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionein promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

“Expression vector” refers to a vector comprising a recombinant polynucleotide
10 expression control sequence operatively linked to a nucleotide sequence to be expressed. An expression vector includes sufficient cis- acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., lentiviruses, retroviruses, adenoviruses, and adeno-associated
15 viruses) that incorporate the recombinant polynucleotide.

Additional promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-100 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that
20 promoter function is preserved when elements are inverted or moved relative to one another, in the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

In order to assess the expression of a CAR polypeptide or portions thereof, the expression
25 vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors; in other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co- transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory
30 sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., 2000 FEBS Letters 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter- driven transcription.

Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. 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). A preferred method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat, Nos. 5,350,674 and 5,585,362.

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome

(e.g., an artificial membrane vesicle). In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (in vitro, ex vivo or in vivo). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma, St. Louis, MO; dicetyl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, NY); cholesterol ("Choi") can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Chloroform is used as the only solvent since it is more readily evaporated than methanol.

"Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 19 1

Glycobiology 5; 505- 10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine- nucleic acid complexes.

5 Regardless of the method used to introduce exogenous polynucleotides into a host cell or otherwise expose a cell to the polynucleotide 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
10 the scope of the disclosure.

Engineered Cell

 In another embodiment, the disclosure provides an engineered cell expressing the
15 chimeric antigen receptor polypeptide described above or polynucleotide encoding for the same, and described above.

 An "engineered cell" means any cell of any organism that is modified, transformed, or manipulated by addition or modification of a gene, a DNA or RNA sequence, or protein or polypeptide. Isolated cells, host cells, and genetically engineered cells of the present disclosure
20 include isolated immune cells, such as NK cells and T cells that contain the DNA or RNA sequences encoding a chimeric antigen receptor or chimeric antigen receptor complex and express the chimeric receptor on the cell surface. Isolated host cells and engineered cells may be used, for example, for enhancing an NK cell activity or a T lymphocyte activity, treatment of cancer, and treatment of infectious diseases.

25 Any cell capable of expressing and/or capable of integrating the chimeric antigen receptor polypeptide, as disclosed herein, into its membrane may be used.

 In an embodiment, the engineered cell includes immunoregulatory cells. Immunoregulatory cells include T-cells, such as CD4 T-cells (Helper T-cells), CD8 T-cells (Cytotoxic T-cells, CTLs), and memory T cells or memory stem cell T cells. In another
30 embodiment, T-cells include Natural Killer T-cells (NK T-cells).

T cells comprise of CD4 and CD8 cells. CD4 is a glycoprotein present on the surface of immune cells such as T helper cells, important in T cell activation and receptor for HIV. Some monocytes or macrophages also express CD4. CD4 is also called OKT4. Cytotoxic T cells are also known as CD8+ T cells or CD8 T cells expressing CD8 glycoprotein at their surfaces. These

5 CD8+ T cells are activated once they are exposed to peptide antigens presented by MHC class I.

In an embodiment, the engineered cell includes NK T cells. NK T cells are well known in the art.

In an embodiment, the engineered cell includes Natural Killer cells. Natural killer cells are well known in the art. In one embodiment, natural killer cells include cell lines, such as NK-92 cells. Further examples of NK cell lines include NKG, YT, NK-YS, HANK-1, YTS cells, and

10 NKL cells.

NK cells mediate anti-tumor effects without the risk of GvHD and are short-lived relative to T-cells. Accordingly, NK cells would be exhausted shortly after destroying cancer cells, decreasing the need for an inducible suicide gene on CAR constructs that would ablate the

15 modified cells.

As used herein, CDXCAR refers to a chimeric antigen receptor having a CDX antigen recognition domain. As used herein CDX may be any one of GD2 and GD3.

TCR deficient T cells used to carry CAR

In one embodiment, engineered cells, in particular allogeneic T cells obtained from

20 donors can be modified to inactivate components of TCR (T cell receptor) involved in MHC recognition. As a result, TCR deficient T cells would not cause graft versus host disease (GVHD).

Sources of Cells

25 The engineered cells may be obtained from peripheral blood, cord blood, bone marrow, tumor infiltrating lymphocytes, lymph node tissue, or thymus tissue. The host cells may include placental cells, embryonic stem cells, induced pluripotent stem cells, or hematopoietic stem cells. The cells may be obtained from humans, monkeys, chimpanzees, dogs, cats, mice, rats, and transgenic species thereof. The cells may be obtained from established cell lines.

30 The above cells may be obtained by any known means. The cells may be autologous, syngeneic, allogeneic, or xenogeneic to the recipient of the engineered cells.

The term “autologous” refer to any material derived from the same individual to whom it is later to be re-introduced into the individual.

The term “allogeneic” refers to any material derived from a different animal of the same species as the individual to whom the material is introduced. Two or more individuals are said to
5 be allogeneic to one another when the genes at one or more loci are not identical. In some aspects, allogeneic material from individuals of the same species may be sufficiently unlike genetically to interact antigenically.

The term “xenogeneic” refers to a graft derived from an animal of a different species.

The term “syngeneic” refers to an extremely close genetic similarity or identity especially
10 with respect to antigens or immunological reactions. Syngeneic systems include for example, models in which organs and cells (e.g. cancer cells and their non-cancerous counterparts) come from the same individual, and/or models in which the organs and cells come from different individual animals that are of the same inbred strain.

In certain embodiments, T and NK cells are derived from human peripheral blood
15 mononuclear cells (PBMC), leukapheresis products (PBSC), human embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs), bone marrow, or umbilical cord blood.

The potential disadvantages of using NK cells in CAR therapy include a lack of persistency that may reduce long-term efficacy.

Finding matching donor T cells for generating CAR T cells could be a challenge as un-
20 matched T cells could attach to the recipient’s tissues, resulting in graft vs. host disease (GVHD).

Recent studies have shown that gene editing through CRISPR-Cas9 for generation of universal CAR T cells may increase cancer risk by creating unintentional mutations and disrupting the function of the p53 repair protein. Given this risk, it is important to seek methods that avoid genome editing when creating a CAR therapy for patients. The natural killer (NK) cell
25 is an ideal platform for creating a universal CAR that avoids risks associated with genome editing. However, the life expectancy of NK CAR cells *in vivo* is very short, with a lifespan of approximately one week

In one embodiment, the present disclosure comprises a method of generating chimeric antigen receptor (CAR)-modified NK cells with long-lived or long persistency in vivo potential
30 for treating a disease. Surprisingly, it is found that CAR NK cells co-expressing IL-15/IL-15sushi or IL-15/IL-15 sushi anchor can extend survival for a long period of time.

In further embodiment, the extension of CAR NK cell survival can be achieved by co-expressing the IL-15/IL-15 anchor.

In some embodiments, CAR NK cells co-expressing IL-15/IL-15sushi or IL-15/IL-15sushi anchor can be scaled up and used as an off-the-shelf product.

5 In one embodiment, CAR NK cells co-expressing IL-15/IL-15 sushi or IL-15/IL-15sushi anchor are capable of continuing supportive cytokine signaling, which is critical to their survival post-infusion in a patient.

In some embodiments, CAR NK T cells co-expressing IL-15/IL-15sushi or IL-15/IL-15sushi anchor can be scaled up and used as an off-the-shelf product.

10 In one embodiment, CAR NK T cells co-expressing IL-15/IL-15 sushi or IL-15/IL-15sushi anchor are capable of continuing supportive cytokine signaling, which is critical to their survival post-infusion in a patient.

In further embodiment, the extension of CAR NK cell survival can be achieved by co-expressing a cytokine selected from a group of IL-7, IL-15, IL-15/IL-15 anchor, IL-15/IL-15RA, 15 IL-12, IL-18 and IL-21.

In one embodiment, CAR NK T cells co-expressing IL-15/IL-15 sushi or IL-15/IL-15sushi anchor are capable of continuing supportive cytokine signaling, which is critical to their survival post-infusion in a patient.

In further embodiment, the extension of CAR NK T cell survival can be achieved by co-expressing a cytokine selected from a group of IL-7, IL-15, IL-15/IL-15 anchor, IL-15/IL-15RA, 20 IL-12, IL-18 and IL-21.

Natural killer T (NK T) cells are a group of T cells that share properties of both T cells and natural killer cells.

In an embodiment, the IL-15 product is modified to create a disulfide bond linking the 25 IL-15/sushi domain complex with an Fc region, such as from IgG1. In this embodiment, the IL-15/sushi complex can be linked to the Fc region, which will form a dimer with a disulfide bridge linking the two molecules. In an embodiment, the leucine at position 52 of the IL-15 is replaced with a cysteine and the serine at position 40 of the Sushi domain is replaced with a cysteine.

In an embodiment, the inducible promoter causes expression upon activation of cellular 30 pathways, such as the T-cell receptor pathway. In this embodiment, expression of the gene of interest will be induced upon activation of the T cell receptor or similar pathways, including

those activated by CARs. Those skilled in the art would understand this to include promoters such as those under the control of nuclear factor of activated T cells (NFAT) promoter including portions of the IL-2 promoter or synthetic promoters consisting of NFAT-binding motifs, the sequences of which are well-described. These are only examples of activation-induced
5 promoters and are not limiting.

uCAR NK cells

The majority of current clinical trials or therapies infuse autologous CAR T cells, as
10 allogeneic CAR T cells are capable of inducing GVHD (graft-versus-host disease) in recipients. Although this autologous approach achieved remarkable clinical successes, the process of manufacturing a patient-specific T cell product is both time-consuming and expensive . Furthermore, it is not always possible to collect enough T cells from a heavily pretreated patient to successfully generate sufficient doses of CAR T cells. There is great demand for the
15 development of an off-the-self allogeneic CAR product. NK cells are similar to T cells in that they are highly cytotoxic immune effectors. In contrast to T cells, NK cells bear the property of killing their targets through an on-specific manner. NK cells can be used as an off-the-self allogeneic product because they usually lack the potential to cause GVHD. The major disadvantage of using NK cells is their lack of persistence *in vivo*, with a half-life of only about a
20 week.

In some embodiments, the present invention discloses a form of universal CAR-expressing NK cells or NK T cells from a healthy donor that can be stored and then infused into an individual on demand. In further embodiments, the invention comprises a method of
25 generating of off-the-self universal CAR NKs from allogeneic healthy donors that can be infused to any patient without causing GVHD.

In some embodiments, NK cell or NK T is obtained from an umbilical cord blood bank and a peripheral blood bank. In a further embodiment, NK is an induced pluripotent stem cell or embryonic stem cell or NK-92 cell.

In some embodiments, the present disclosure comprises a method for having a CAR or compound CAR (cCAR) co-expressing IL-15/IL-15sushi in a NK cell. These engineered NK cells are called uCAR NK cells.

In some embodiments, uCAR NK cells have CAR or cCAR co-expressing IL-15/IL-15sushi. In further embodiments, uCAR NK cells is capable of persisting for more than one week *in vivo*.

In some embodiments, the present disclosure comprises a method for a uCAR NK cell with a vector expressing a CAR or cCAR with IL-15/IL-15sushi.

In some embodiments, co-expression of IL-15/IL-15sushi with a CAR or cCAR provides long-term persistence for a NK cell in a subject.

In some embodiments, co-expression of IL-15/IL-15sushi with a CAR or cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or by recruiting innate immune cells to cancer cells.

In some embodiments, the present disclosure comprises a method for generating a NK cell with one CAR or cCARs co-expressing IL-15/IL-15sushi. In further embodiments, a particular tumor antigen targeted by an antigen recognition domain in a CAR can be selected from the group of, but not limited to: GD2, GD3, interleukin 6 receptor, FSHR, ROR1, PSMA, PSCA (prostate stem cell antigen), MAGE A3, Glycolipid, glypican 3, F77, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE-6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, MUC1, MUC2, MUC3, MUC4, MUC5, MMG49 epitope, CD30, EGFRvIII, CD33, CD123, CLL-1, NKG2D, NKG2D receptors, immunoglobulin kappa and lambda, CD38, CD52, CD47, CD200, CD70, CD56, CD19, CD20, CD22, CD38, BCMA, CS1, BAFF receptor, TACI, CD3, CD4, CD8, CD5, CD7, CD2, and CD138.

In some embodiments, the present disclosure comprises a method for the treatment of a disorder or disease by the infusion of a therapeutically effective amount of NK cells that are genetically engineered to express IL-15/IL-15sushi and/or a CAR with an antigen recognition domain for a particular tumor antigen. In further embodiments, a particular tumor antigen targeted by an antigen recognition domain can be selected from the group of, but not limited to: GD2, GD3, interleukin 6 receptor, FSHR, ROR1, PSMA, PSCA (prostate stem cell antigen), MAGE A3, Glycolipid, glypican 3, F77, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE-6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1,

MUC1, MUC2, MUC3, MUC4, MUC5, MMG49 epitope, CD30, EGFRvIII, CD33, CD123, CLL-1, NKG2D, NKG2D receptors, immunoglobulin kappa and lambda, CD38, CD52, CD47, CD200, CD70, CD56, CD19, CD20, CD22, CD38, BCMA, CS1, BAFF receptor, TACI, CD3, CD4, CD8, CD5, CD7, CD2, and CD138.

5 In some embodiments, the administration of a high dose of uCAR NK cells can cause cytokine release syndrome (CRS). In present disclosure comprises a method of reduction or avoidance of CRS by providing a subject with a lower doses or split doses of uCAR NK cells.

10 **Suicide and safety switch systems**

The engineered cells of the present disclosure may also include a suicide system. Suicide systems provide a mechanism whereby the engineered cell, as described above, may be deactivated or destroyed. Such a feature allows precise therapeutic control of any treatments wherein the engineered cells are used. As used herein, a suicide system provides a mechanism by which the cell having the suicide system can be deactivated or destroyed. Suicide systems are well known in the art.

In one embodiment, a suicide system includes a gene that can be pharmacologically activated to eliminate the containing cells as required. In specific aspects, the suicide gene is not immunogenic to the host harboring the polynucleotide or cell. In one example, the suicide system includes a gene that causes CD20 to be expressed on the cell surface of the engineered cell. Accordingly, administration of rituximab may be used to destroy the engineered cell containing the gene.

In some embodiments, the suicide system includes an epitope tag. Examples of epitope tags include a c-myc tag, CD52 streptavidin-binding peptide (SBP), and truncated EGFR gene (EGFRt). In this embodiment, the epitope tag is expressed in the engineered cell. Accordingly, administration of an antibody against the epitope tag may be used to destroy the engineered cell containing the gene.

In another embodiment, the suicide system includes a gene that causes truncated epidermal growth factor receptor to be expressed on the surface of the engineered cell. Accordingly, administration of cetuximab may be used to destroy the engineered cell containing the gene.

In another embodiment, the suicide system includes CD52 to be expressed on the surface of the engineered cell. Accordingly, administration of anti-52 monoclonal antibody (CAMPATH, alemtuzumab) may be used to destroy the engineered cell containing the gene.

In another embodiment, the suicide system includes CAMPATH (alemtuzumab).

5 Accordingly, administration of anti-52 monoclonal antibody (CAMPATH) may be used to destroy the engineered cell without expressing a tag or a gene as CAR T cells or T cells highly express CD52.

In another embodiment, the suicide gene may include caspase 8 gene, caspase 9 gene, thymidine kinase, cytosine deaminase (CD), or cytochrome P450.

10 Examples of further suicide systems include those described by Jones et al. (Jones BS, Lamb LS, Goldman F and Di Stasi A (2014) Improving the safety of cell therapy products by suicide gene transfer. Front. Pharmacol. 5:254. doi: 10.3389/fphar.2014.00254), which is herein incorporated by reference in its entirety.

15 **Compound CAR (cCAR)**

As used herein, a compound CAR (cCAR) or multiple CAR refers to an engineered cell having at least two complete and distinct chimeric antigen receptor polypeptides. As used herein, a “distinct chimeric antigen receptor polypeptide” has a unique antigen recognition domain, a signal peptide, a hinge region, a transmembrane domain, at least one costimulatory domain, and a signaling domain. Therefore, two unique chimeric antigen receptor polypeptides will have different antigen recognition domains. The signal peptide, hinge region, transmembrane domain, at least one costimulatory domain, and signaling domain may be the same or different between the two distinct chimeric antigen receptor polypeptides. As used herein, a chimeric antigen receptor (CAR) unit refers to a distinct chimeric antigen receptor polypeptide, or a polynucleotide encoding for the same.

25 As used herein, a unique antigen recognition domain is one that is specific for or targets a single target, or a single epitope of a target.

As used herein, in the context of compound CAR. A single chimeric antigen receptor polypeptide has only one unique antigen recognition domain. By way of further explanation, this single antigen recognition domain recognizes and binds to a single antigen or a single antigen epitope only.

In some embodiments, the compound CAR targets the same antigen. For example, cCAR targets different epitopes or parts of a single antigen. In some embodiments, each of the CAR units present in the compound CAR targets different antigen specific to the same or different disease condition or side effects caused by a disease condition.

5 In some embodiments, the compound CAR targets two different antigens.

Creation of compound CARs bearing different CAR units can be quite challenging: (1) CAR-CAR interactions might have a deleterious effect and an appropriate CAR design is a key to offset this effect; (2) a compound CAR in a single construct could increase the length of the expression cassette, which may cause the reduction of the viral titer and level of protein
10 expression; (3) an appropriate design to include various CAR body elements particularly to select a strategy to express multiple CARs in a single vector is required; (4) A strong promoter is particularly important for a compound CAR that bears additional units of CAR; (5) The hinge region in the CAR needs to be designed so that interaction of the hinge region between each CAR unit is avoided preferably; (6) two or more units of CARs expressing in a cell may cause
15 toxic effects (CAR-CAR interaction). Applicants herein provide novel and surprising CAR compositions and methods to overcome these hurdles.

The transduction efficiency (percentage of CAR T cells) for cCARs is often lower than for a single-unit CAR. There are several ways to improve efficiency, at both the transfection and transduction steps. To improve viral titer for making cCARs, it is preferred to use LentiX™ 293
20 T (Clontech/Takara) packaging cell line, which is selected for high titer lentivirus production, instead of the commonly used HEK-293FT. It is also preferable to increase the amount of plasmid DNA (containing the cCAR construct) 1.5- to 2.0-fold when transfecting packaging cells, to increase transfection efficiency. The amount of viral packaging plasmids and transfection reagent remains the same during the forming of complexes. Transduction efficiency
25 can be further enhanced by lowering the ratio of T cells to viral vector during the transduction step, to 0.3×10^6 cells per mL, and increasing the volume of lentiviral supernatant or lentiviruses.

In one embodiment, the present disclosure provides an engineered cell having multiple CAR units. This allows a single engineered cell to target multiple antigens. Targeting multiple surface markers or antigens simultaneously with a multiple CAR unit prevents selection of
30 resistant clones and reduces tumor recurrence. Multiple CAR T cell immunotherapies, with each

individual component CAR comprising various domains and activation sites has not yet been developed for any malignancies.

In one aspect of the present disclosure, cCAR includes multiple CAR units. In some embodiments, cCAR includes at least two CAR units. In another embodiment, the cCAR
5 includes at least three CAR units. In another embodiment, the cCAR includes at least four units.

In one embodiment, the present disclosure provides an engineered cell having at least two distinct chimeric antigen receptor polypeptides, each having a different antigen recognition domain.

In one embodiment, the engineered cell having at least two distinct chimeric antigen
10 receptor polypeptides is a T-cell or NK T-cell. The T-cell may be engineered so that it does not express a cell surface antigen. For example, a T-cell may be engineered so that it does not express a CD45 cell surface antigen.

In a preferred embodiment, the engineered cell having at least two distinct chimeric antigen receptor polypeptides is a primary NK cell or NK T cell isolated from the peripheral
15 blood or cord blood and NK-92 cells, such that it is administered “off-the-shelf” to any mammal with a disease or cancer.

In one embodiment, the engineered cell includes (i.) a first chimeric antigen receptor polypeptide comprising a first antigen recognition domain, a first signal peptide, a first hinge region, a first transmembrane domain, a first co-stimulatory domain, and a first signaling
20 domain; and (ii.) a second chimeric antigen receptor polypeptide comprising a second antigen recognition domain, a second signal peptide, a second hinge region, a second transmembrane domain, a second co-stimulatory domain, and a second signaling domain. The first antigen recognition domain is different from the second antigen recognition domain.

In a preferred embodiment, each engineered CAR unit polynucleotide has different
25 nucleotide sequences in order to avoid homologous recombination.

In one embodiment, the target of the first antigen recognition domain is selected from the group of, but not limited to, GD2, GD3, interleukin 6 receptor, ROR1, PSMA, PSCA (prostate stem cell antigen), MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP,
30 ErbB, c-Met, MART-1, MUC1, MUC2, MUC3, MUC4, MUC5, CD30, EGFRvIII, CD33, CD123, CLL-1, NKG2D, NKG2D receptors, immunoglobulin kappa and lambda, CD38, CD52,

CD19, CD20, CD22, CD38, BCMA, CS1, BAFF receptor, TACI, CD3, CD4, CD8, CD5, CD7, CD2, and CD138; and the target of the second recognition domain is selected from the group consisting of GD2, GD3, interleukin 6 receptor, ROR1, PSMA, PSCA (prostate stem cell antigen), MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, MUC1, MUC2, MUC3, MUC4, MUC5, CD30, EGFRvIII, CD33, CD123, CLL-1, NKG2D, NKG2D receptors, immunoglobulin kappa and lambda, CD38, CD52, CD19, CD20, CD22, CD38, BCMA, CS1, BAFF, BAFF receptor, April receptor, TACI, CD3, CD4, CD8, CD5, CD7, CD2, and CD138.

In one embodiment, the target of the first antigen recognition domain is selected from the group of, but not limited to: GD2, GD3, CD19, CD20, CD22, CD38, CD138, BCMA, CS1, BAFF, BAFF receptor, TACI, April, April receptor, CD3, CD4, CD5, CD7, CD2, CLL-1, CD33, CD123, NKG2D receptors and CD30; the target of the second recognition domain is selected from a group consisting of GD2, GD3, CD19, CD20, CD22, CD38, CD138, BCMA, CS1, BAFF, April, April receptor, BAFF receptor, TACI, CD3, CD4, CD5, CD7, CD2, CLL-1, CD33, CD123, NKG2D receptors and CD30.

In one embodiment, each CAR unit includes the same or different hinge region. In another embodiment, each CAR unit includes the same or different transmembrane region. In another embodiment, each CAR unit includes the same or different intracellular domain.

In one embodiment, each CAR unit includes the CD3 zeta chain signaling domain.

In one embodiment, each distinct CAR unit includes different co-stimulatory domains. For example, the first chimeric antigen receptor polypeptide includes a 4-1BB co-stimulatory domain; and the second chimeric antigen receptor polypeptide includes a CD28 co-stimulatory domain.

In one embodiment, each distinct CAR unit includes the same co-stimulatory domains. For example, the first chimeric antigen receptor polypeptide includes a 4-1BB co-stimulatory domain; and the second chimeric antigen receptor polypeptide includes a 4-1BB co-stimulatory domain.

In another embodiment, the hinge region is designed to exclude amino acids that may cause undesired intra- or intermolecular interactions. For example, the hinge region may be designed to exclude or minimize cysteine residues to prevent formation of disulfide bonds. In

another embodiment, the hinge region may be designed to exclude or minimize hydrophobic residues to prevent unwanted hydrophobic interactions.

Compound CAR can perform killing independently or in combination. Multiple or compound CAR comprises same or different hinge region, same or different transmembrane, same or different co-stimulatory and same or different intracellular domains. Preferably, the hinge region is selected to avoid the interaction site.

The compound CAR of the present disclosure may target same or different tumor populations in T or NK cells. The first CAR, for example, may target the bulky tumor population and the next or the second CAR, for example, may eradicate cancer or leukemic stem cells, to avoid cancer relapses.

In accordance with the present disclosure, it was surprisingly found that the compound CAR in a T or NK cells targeting different or same tumor populations combat tumor factors causing cancer cells resistant to the CAR killing activity, thereby producing down regulation of the target antigen from the cancer cell surface. It was also surprisingly found that this enables the cancer cell to “hide” from the CAR therapy referred to as “antigen escape” and tumor heterogeneity, by which different tumor cells can exhibit distinct surface antigen expression profiles. As present disclosure below, it is surprisingly found that the compound CAR has significant advantages over single-CAR therapies due to its multi-targeting ability. While loss of a single antigen under antigen-specific selection pressure is possible, loss of two major antigens simultaneously is much less likely.

In one embodiment, the antigen recognition domain includes the binding portion or variable region of a humanized monoclonal or humanized polyclonal antibody directed against (selective for) the target.

In one aspect to the invention, an antigen recognition domain can be a bispecific tandem chimeric antigen receptor that includes two targeting domains. In further embodiment, there is a multispecific tandem chimeric antigen receptor that includes three or more targeting domains.

In certain aspects to the invention, an antigen recognition domain can be a bispecific chimeric antigen receptor (derived from a bispecific antibody) that includes two targeting domains.

In one embodiment, a bispecific tandem chimeric antigen receptor or a bispecific chimeric antigen receptor effectively offsets tumor escape or antigen loss and increases the sensitivity of antigen recognition.

5 In another embodiment, the antigen recognition domain includes camelid single domain antibody, or portions thereof. In one embodiment, camelid single-domain antibodies include heavy-chain antibodies found in camelids, or VHH antibody. A VHH antibody of camelid (for example camel, dromedary, llama, and alpaca) refers to a variable fragment of a camelid single-chain antibody (See Nguyen et al, 2001; Muyldermans, 2001) and also includes an isolated VHH antibody of camelid, a recombinant VHH antibody of camelid, or a synthetic VHH antibody of
10 camelid.

Enhancers for CAR functions and promotion of T and innate cell expansion or proliferation

15 IL-15/IL15sushi enhancer

In one embodiment, A CAR construct with IL-15/IL15sushi enhancer is shown in figure 53, A CAR is equipped with secreting IL-15/IL-15sushi complexes. A CAR with IL-15/IL-15sushi is linked with the P2A self-cleaving sequence. The IL-15/IL-15sushi portion is composed
20 of IL-2 signal peptide fused to IL-15 and linked to the sushi domain of IL-15 alpha receptor via a 26-amino acid poly-proline linker. CAR has scFv, costimulatory domain (including, but not limited to CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain. The IL-15 signal peptide in the IL-15 is replaced with IL-2 signal peptide (leader sequence), a strong signal peptide to provide a high efficiency of IL-15/IL-15sushi secretion. The peptide self cleavage peptides of
25 the construct may include, but is not limited to, P2A, T2A, F2A and E2A. The secreting enhancer (s) of the construct may also include, but is not limited to, IL-15/IL-15sush, IL-15, IL-21, IL-18, IL-7 and IL-12. The secreting enhancer, such as IL-15/IL-15sushi enhances CAR T or NK cell expansion and persistency. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their
30 neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion are stable and enhances CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and

anti-tumor activity . The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

The IL-15 can be a variant, IL-15N72D described in elsewhere, US8507222 B2.

5

IL-15/IL15sushi anchor enhancers

In one embodiment, a CAR construct with IL-15/IL-15sushi anchor is shown in Figure 54. A CAR IL-15/IL15sushi anchor construct consists a SFFV promoter driving the expression of a CAR and an IL-15/IL-15sushi anchor (also called anchor) linked by a P2A peptide. Upon
10 cleavage of this P2A peptide, IL-15/IL-15 anchor CAR splits to a CAR and an IL-15/IL-15sushi anchor. The IL-15/IL-15sushi portion of anchor is composed of IL-2 signal peptide fused to IL-15 and linked to sushi domain of IL-15 alpha receptor via a 26-amino acid poly-proline linker. Both CAR and anchor comprise a hinge (H) region, a transmembrane domain (TM). CAR also has scFv, costimulatory domain (including, but not limited to CD28 or 4-1BB) and intracellular
15 signaling, CD3 zeta chain while anchor does not bear these components. IL-15/IL-15sushi anchor provides a synergistic effect of T cell activation or anti-tumor activity with CD28 or 4-1BB. CAR is more powerful when equipped with IL-15/IL-15sushi anchor (Figure 54)

The IL-15 can be a variant, IL-15N72D described in elsewhere, US8507222 B2

4-1BBL enhancer

20 In another embodiment, a CAR construct with a 4-1BBL enhancer is shown in Figure 55. A CAR 4-1BBL construct consists a SFFV promoter driving the expression of a CAR and an enhancer, 4-1BBL (CD137L) linked by a P2A peptide. Upon cleavage of this P2A peptide, A CAR construct with 4-1BBL splits to a CAR and the full length of 4-1BBL protein. A CAR comprises a leader sequence and scFv, a hinge (H) region, a transmembrane domain (TM). CAR
25 also has costimulatory domain (including, but not limited to, CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain while 4-1BBL does not bear these components. 4-1BBL provides a synergistic effect of T cell activation or anti-tumor activity with CD28 or 4-1BB. CAR is more powerful when equipped with 4-1BBL.

30

IL-15 enhancer

A CAR function can be enhanced by incorporating a secreting enhancer, IL-15 shown in Figure 56. A CAR 4-IL-15 construct consisted a SFFV promoter driving the expression of a CAR and an enhancer, IL-15 linked by a P2A peptide. Upon cleavage of this P2A peptide, A CAR construct with IL-15 splits to a CAR and the full length of IL-15 protein. A CAR comprises
 5 a leader sequence and scFv, a hinge (H) region, a transmembrane domain (TM). CAR also has costimulatory domain (including, but not limited to, CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain while IL-15 does not bear these components. Secreting IL-15 provides a synergistic effect of T cell activation or anti-tumor activity with CD28 or 4-1BB. CAR is more powerful when secreting IL-15. The IL-15 signal peptide in the IL-15 was replaced with IL-2
 10 signal peptide (leader sequence), a strong signal peptide to provide a high efficiency of IL-15 secretion.

A CAR with multiple enhancers

An example for generation of a CAR with multiple enhancers (CAR super). Figure 57, a schematic showing a CAR enhancer construct. The construct consists a SFFV promoter driving
 15 the expression of a CAR and enhancers, 4-1BBL (CD137L) and IL-15/IL-15sushi linked by a P2A and T2A peptide, respectively. Upon cleavage of this P2A and T2A peptides, a CAR construct with 4-1BBL and IL-15/IL-15sushi splits to a CAR and the full length of 4-1BBL protein, and secreting IL-15/IL-15sushi. A CAR comprises a leader sequence and scFv, a hinge
 20 (H) region, a transmembrane domain (TM). CAR also has costimulatory domain (including, but not limited to, CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain. 4-1BBL ligand provides a synergistic effect of T or NK cell activation or anti-tumor activity with CD28 or 4-1BB (but not limited to). The peptide self cleavage peptides of the construct may include, but is not limited to, P2A, T2A, F2A and E2A. The secreting enhancer (s) of the construct may also
 25 include, but is not limited to, IL-15/IL-15sushi, IL-15, IL-21, IL-18, IL-7 and IL-12. The secreting enhancer, such as IL-15/IL-15sushi enhances CAR T or NK cell expansion and persistency. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. . The soluble IL-15/IL-15sushi fusion are stable and enhances CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-
 30 15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

BCMA-CS1 compound CAR (BCMA-CS1 cCAR)

Multiple myeloma (MM) is a blood cancer caused by the unusually rapid proliferation of plasma cells and accounts for 18% of all blood cancers in the United States. Treatment options for MM include chemotherapy, corticosteroid therapy, targeted therapy, high-dose chemotherapy with stem cell transplant, biological therapy, radiation therapy, monoclonal antibodies, proteasome inhibitors, and surgery. Even with these available treatments, the five-year survival rate for MM remains at 49.6%. However, there remains no cure for MM, and nearly all patients relapse after treatment.

Current CAR technology efforts in multiple myeloma involve the use of a BCMA (CD269) targeted CART-cell against bulk disease spearheaded by James Kochenderfer (NIH). Those patients in remission after BCMA CAR treatment eventually relapse and this may be due to the fact that some myeloma cells are dim (weak) or negative expression for BCMA. Therefore, a single target for CAR based treatment may not be sufficient to prevent myeloma relapse. CS1 (SLAMF7) is another good target for myeloma as its expression is typically high and uniform in myeloma cells as well as being implicated in myeloma cell adhesion and tumorigenicity.

The present disclosure is composed of a single CAR T-cell expressing 2 discrete CAR units in a vector with independent signaling domains can be utilized as a novel approach for targeting multiple antigens and potentially avoiding tumor relapse. A compound CAR (cCAR) comprising of a BCMA CAR linked to a CS1 CAR via a self-cleaving P2A peptide and expressed both functional CAR molecules on the surface of a T cell.

In the present disclosure, it was surprisingly found that this BCMA-CS1 cCAR (BC1cCAR) T-cell exhibits potent and specific anti-tumor activity in vitro, as well as controlling significant tumor growth in vivo. We demonstrate, for the first time, a 2-unit discrete CAR is able to target effectively both antigens in vitro, with potential implications for more comprehensive clinical outcomes. It is unexpected that targeting multiple myeloma with a compound CAR targeting both BCMA and CS1 in combination is a very strong strategy. This novel approach circumvents the antigen escape (loss of a single antigen) from selection pressure of single CAR treatment due to combinatorial pressure from a compound design.

BCMA (B-cell maturation antigen) and CS1 (SLAMF7) were preferably chosen as targets for our compound CAR because the vast majority of myeloma cases express either or both surface antigens, and these antigens do not include hematopoietic stem cells. The use of two different targets widely expressed on plasma cells, BCMA and CS1, can increase coverage and efficaciously eradicate cancerous cells to prevent antigen escape

In this disclosure, it is surprisingly found that the addition of CS1 as a target to the BCMA CAR enhanced the anti-tumor response by eliminating surviving BCMA⁻CS1⁺ myeloma cells to reduce the risk of relapse. BCMA and CS1 (CD319) are both widely expressed on MM cells, and this high expression allows the BCMA-CS1 cCAR to have a comprehensive coverage of all potentially cancerous cells. This allows for a more complete elimination of cancerous cells to reduce antigen escape by hitting hard with multiple targets simultaneously before resistance develops.

In one embodiment, BCMA-CS1 directed BCMA-CS1cCAR (BC1cCAR) therapy is as a “bridge” to bone marrow transplant (BMT) or combination with a heavy chemotherapy plus BMT. BCMA-CS1 cCAR can offer a path to a potentially curative BMT option to many patients that previously would have a residual disease. Current literature supports the idea that reducing the minimal residual disease burden (MRD) to an undetectable level could be associated with improved patient outcomes. This could be extremely beneficial in terms of prevention of relapse for the difficult to treat and highly aggressive malignancies.

In another embodiment, BCMA-CS1 cCAR therapy is able to bring down disease burden to the lowest possible level prior to transplant or thoroughly eliminate MRD, it can be expected that the relapse rate will decrease and long-term disease-free survival rate will increase, and patient outcomes will be dramatically improved.

In one embodiment, BCMA-CS1 cCAR therapy can have further applications for patients with BCMA⁺ and/or CS1⁺ multiple myelomas beyond a bridge to bone marrow transplantation. BCMA-CS1cCAR therapy as a standalone therapy, or as a part of a patient-individualized immuno-chemotherapy regimen. For elderly patients, or for those with comorbidities who cannot tolerate highly intensive chemotherapy or BMT, this might be a promising strategy to prolong patient’s survival time and reserve better life quality.

In some embodiments, BCMA-CS1cCAR T-cell therapy can be developed as a “bridge to transplant,” a supplement to chemotherapy, or as a standalone therapy for patients with multiple myeloma.

5 In some embodiments, the present disclosure provides a compound CAR polypeptide engineered cell that targets cells expressing BCMA or CS1 antigens or both. The targeted cells may be cancer cells, such as, but not limited to, lymphomas, or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms are selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom’s macroglobulinema, heavy chain diseases, solitary bone plamacytoma, monoclonal gammopathy
10 of undetermined significance (MGUS) and smoldering multiple myeloma.

It was surprised to find that co-expression of IL-15/IL-15sushi with cCAR could provide long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

15 Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi anchor or 4-1BBLwith BCMA-CS1 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

20 Without wishing to be bound by theory, it is believed that co-expression of IL-21 or IL-21 anchor with BCMA-CS1 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a BCMA-CS1 cCAR polypeptide and IL-15/IL-15sushi (SEQ ID NO. 42), and corresponding nucleotides (SEQ ID NO. 43).
25

BCMA1-BCMA2 compound CAR (BCMA1-BCMA2 cCAR) (Figure 38)

Initial remission of most B-ALL can be seen in CD19 CAR T therapy but relapses with epitope loss occur in 10% to 20% of responders.

30 Current CAR technology efforts in multiple myeloma involve the use of a BCMA (CD269) targeted CAR T-cell against multiple myeloma spearheaded by James Kochenderfer

(NIH). Those patients in initial remission after BCMA CAR treatment eventually relapse and this may due to the fact that some myeloma cells are dim (weak) or negative expression for BCMA. In addition, potency of a single CAR is also an issue for eliminating multiple myeloma cells in the patients. Therefore, a single target for CAR based treatment may not be sufficient to prevent myeloma relapse.

In one embodiment, the antibody recognition domain includes the binding variable region of a monoclonal antibody, single chain fragment variable (scFv). The scFv includes one light and heavy of antibody. In a particular embodiment, antigen recognition domain is composed of two different heavy chain domains (VHH). Each heavy chain domain binds to a different epitope of the same antigen or different antigen. A VHH antibody is more stable and robust than a whole antibody.

In some embodiments, the compound CAR targets the same antigen. For example, cCAR targets different epitopes or parts of a single antigen. In some embodiments, each of the CAR units present in the compound CAR targets different epitopes specific to the same antigen but different locations.

In some embodiments, a compound CAR targets different epitopes on one antigen.

The present disclosure is composed of a single CAR T-cell expressing two discrete CAR units in a vector with independent signaling domains can be utilized as a novel approach for targeting different epitopes on one antigen, and potentially avoiding tumor epitope skipping or epitope loss or epitope escape. A compound cCAR (BCMA1-BCMA2 cCAR) is comprising of one BCMA CAR (BCMA1 CAR) linked to another BCMA CAR (BCMA2 CAR) via a self-cleaving P2A peptide and expressed both functional CAR molecules on the surface of a T cell. Both units of CARs in cCAR target the same antigen, BCMA.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a BCMA antigen recognition epitope and second chimeric antigen receptor polypeptide having a different BCMA recognition epitope. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 3 and corresponding polynucleotide of SEQ ID NO. 4.

In the present disclosure, it was surprisingly found that this BCMA1-BCMA2 cCAR T-cell exhibits potent and specific anti- tumor activity in vitro, as well as controlling significant tumor growth in vivo. We demonstrate, for the first time, a 2-unit discrete CAR is able to target

effectively both different epitopes on one antigen, BCMA in vitro, with potential implications for more comprehensive clinical outcomes. It is unexpected that targeting multiple myeloma with a compound CAR targeting different epitopes in combination is a very strong strategy. This novel approach circumvents the epitope escape (loss of a single epitope or epitope skipping) from selection pressure of single CAR treatment due to combinatorial pressure from a compound design.

In this disclosure, it is surprisingly found that the addition of epitope as a target to the BCMA CAR enhances the anti-tumor response and reduces the risk of multiple myeloma relapse due to the loss of BCMA epitope.

In one embodiment, BCMA1-BCMA2 directed therapy is as a “bridge” to bone marrow transplant (BMT) or combination with a heavy chemotherapy plus BMT. BCMA1-BCMA2 cCAR can increase the sensitivity of recognition of BCMA antigen, and offer a path to a potentially curative BMT option to many patients that previously would have a residual disease. Current literature supports the idea that reducing the minimal residual disease burden (MRD) to an undetectable level could be associated with improved patient outcomes. This could be extremely beneficial in terms of prevention of relapse for the difficult to treat and highly aggressive malignancies.

In another embodiment, BCMA1-BCMA2 cCAR therapy is able to bring down disease burden to the lowest possible level prior to transplant or thoroughly eliminate MRD, it can be expected that the relapse rate will decrease and long-term disease-free survival rate will increase, and patient outcomes will be dramatically improved.

In some embodiments, the present disclosure provides a compound CAR polypeptide engineered cell that targets two different epitopes on the BCMA antigen. The targeted cells may be cancer cells, such as, but not limited to, lymphomas, or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms are selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom’s macroglobulinema, heavy chain diseases, solitary bone plamacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma.

Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi or IL-15/IL-15sushi anchor or 4-1BBLwith BCMA1-BCMA2 cCAR provides long-term

durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

Without wishing to be bound by theory, it is believed that co-expression of IL-21 or IL-21 anchor with BCMA1-BCMA2 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

CD123-CD33 compound CAR (CD123-CD33 cCAR)

Translating CAR success to AML requires a careful understanding of characteristics unique to the disease. AML is characterized by the presence of blast cells, which are highly aggressive and rapidly dividing cells that form the bulk of disease. Unlike B-cell malignancies, AML is uniquely challenging to treat due to the role of leukemic stem cells (LSCs). LSCs are a population of cells expressing markers of hematopoietic stem cells (CD34+CD38-) that are capable of initiating and maintaining hematopoietic malignancy, producing clonal cell populations that overtake healthy bone marrow. Since LSCs remain mostly in the quiescent phase of the cell cycle, chemotherapy directed against rapidly dividing tumor populations leaves LSCs untouched. Most often it is this elusive population that comprises minimal residual disease (MRD) and is responsible for inevitable relapse after AML treatment. Successful translation of CAR therapy to AML to completely eliminate disease and ensure no relapse requires careful antigen selection that will enable eradication of not just bulk leukemic disease, but also leukemic stem cells.

It is expected that a CD123-CD33 cCAR that will ablate both CD33+ and CD123+ cells without causing a CAR and CAR interaction. A useful analogy in this case would be to consider AML as a cancer tree with leaves and roots. While the leaves make up the majority/bulk of the disease (these are the CD33+ AML blast cells), trimming these leaves does not prevent the tree from growing further unless you also pull the tree from its root (these are the CD123+CD34+CD38- LSCs). A study of 319 AML patients and found that 87.8% of cases expressed CD33, so it follows that targeting CD33 might most leukemic cells. However, patients treated with gentuzumab ozogamicin, an anti-CD33 antibody therapy linked to calicheamicin, relapsed with CD33+ AML likely due to acquired chemoresistance to calicheamicin. Therefore, while targeting CD33 eliminates the majority of disease, the chemoresistant LSCs must also be

targeted or relapse will occur. This can be achieved by targeting CD123, which is overexpressed on CD34+CD38- LSCs as compared to healthy hematopoietic stem cells. Considering that 97.2% of AML cases express at least one of the two targets, targeting both CD123 and CD33 would therefore eliminate all cancer cells in the majority of patients, increasing treatment efficacy and uprooting the cancer tree.

AML is a rapidly progressing blood cancer that accounts for about 15-20% of acute childhood leukemias and 80% of acute adult leukemia cases. Patients are nowadays still treated by high-dose multi-agent chemotherapy potentially followed by hematopoietic stem cell transplantation. Despite such intensive therapies, which are often associated with considerable toxicities and even death, about 60-70% of AML patients still relapse due to acquired therapy resistance or LSC re-emergence. Moreover, the five-year survival rate from AML remains at a dismal 27%. However, there are a limited number of clinical trials attempting the use of CARs to treat AM.

The present disclosure is composed of a single CAR T-cell expressing two discrete CAR units in a vector with independent signaling domains can be utilized as a novel approach for targeting multiple antigens and potentially avoiding tumor relapse. A compound CAR (cCAR) comprising of a CD123 CAR linked to a CD33 CAR via a self-cleaving P2A peptide and expressed both functional CAR molecules on the surface of a T cell.

In the present disclosure, it was surprisingly found that this CD123-CD33 cCAR T-cell exhibits potent and specific anti- tumor activity in vitro, as well as controlling significant tumor growth in vivo. We demonstrate, for the first time, a 2-unit discrete CAR is able to target effectively both antigens in vitro, with potential implications for more comprehensive clinical outcomes. It is unexpected that targeting AML with a compound CAR targeting both CD123 and Cd33 in combination is a very strong strategy. This novel approach circumvents disease relapses associated with LSCs, and antigen escape (loss of a single antigen) from selection pressure of single CAR treatment due to combinatorial pressure from a compound design.

In this disclosure, it is surprisingly found that the addition of CD123 as a target to the CD33 CAR enhanced the anti-tumor response by eliminating both leukemic blasts and its root, LSCs to reduce the risk of relapse. This allows for a more complete elimination of cancerous cells to reduce disease relapse by deleting both slowly growing LSCs and proliferative leukemic cells.

In this disclosure, it is surprisingly found that CD123-CD33 cCAR T-cells are able to eliminate regular leukemic cells and leukemic precursor cells to reduce the risk of relapse, and enhance anti-tumor activities.

In this disclosure, it is also surprisingly found that CD123-CD33 cCAR T-cells exhibit
5 a more complete elimination of cancerous cells to reduce antigen escape by hitting hard with multiple targets simultaneously before resistance develops.

In one embodiment, CD123-CD33cCAR T-cell therapy could be developed as a “bridge to transplant”, a supplement to chemotherapy, or a checkpoint blockage (including, but not limited to PD-L1, CTLA-4 inhibitor) or as a standalone therapy for patients with diseases
10 including, but not limited to, acute myeloid leukemia, myelodysplastic syndromes, chronic myeloid leukemia and chronic myeloproliferative disorders.

In another embodiment, CD123-CD33cCAR T-cell therapy can use to bring down disease burden to the lowest possible level prior to transplant or thoroughly eliminate MRD, it can be expected that the relapse rate will decrease and long-term disease-free survival rate will
15 increase, and patient outcomes will be dramatically improved.

In one embodiment, CD123-CD33cCAR T-cell therapy can have further applications for patients with Cd123+ and/or CD33+ leukemic patients beyond a bridge to bone marrow transplantation. CD123-CD33cCAR T-cell therapy as a standalone therapy, or as a part of a patient-individualized immuno-chemotherapy regimen. For elderly patients, or for those with
20 comorbidities who cannot tolerate highly intensive chemotherapy or BMT, this might be a promising strategy to prolong patient’s survival time and reserve better life quality.

Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi or IL-15/IL-15sushi anchor or 4-1BBLwith CD123-CD33 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer
25 cells or recruiting innate immune cells to cancer cells.

Without wishing to be bound by theory, it is believed that co-expression of IL-21 or IL-21 anchor with CD123-CD33 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a CD123-CD33 cCAR polypeptide and IL-15/IL-15sushi (SEQ ID NO. 34), and corresponding nucleotides (SEQ ID NO. 35).

CLL-1-CD33 compound CAR (CLL-1-CD33 cCAR)

A cCAR contains two units of CARs, CLL-1CAR and CD33 CAR targeting tumor cells
5 expressing CLL-1 and CD33, respectively. CD33b CAR and CLL-1 CAR were used to
construct a version of cCAR shown in figure 92. The construct comprises a SFFV promoter
driving the expression of multiple modular units of CARs linked by a P2A peptide. Upon
cleavage of the linker, the cCARs split and engage upon targets expressing CD33 and CLL-1.
The activation domains of the construct included 4-1BB on the CD33b (CD33) CAR unit and a
10 CD28 on the CLL-1 CAR unit. This CD33b-CLL-1 cCAR was designed to delete myeloid
leukemic cells including leukemic stem cells.

At the present, therapies for MDS, MPN (chronic myeloproliferative neoplasms) and
AML have focused on the leukemic blast cells because they are very abundant and clearly
represent the most immediate problem for patients. Importantly, leukemic stem cells (LSCs) are
15 quite different from most of the other leukemia cells (“blast” cells), and they constitute a rare
subpopulation. While killing blast cells can provide short-term relief, LSCs, if not destroyed, will
always re-grow, causing the patient to relapse. It is imperative that LSCs be destroyed in order to
achieve durable cures for MDS disease. Unfortunately, standard drug regimens are not effective
against MDS or MPN or AML LSCs. Therefore, it is critical to develop new therapies that can
20 specifically target both the leukemic stem cell population and the bulky leukemic population.
The compound CAR disclosed in the present disclosure target both populations and is embodied
herein.

In one aspect of the present disclosure, CLL-1 antigen is one of the targets for cCAR
therapy. C-type lectin-like- 1 (CLL-1) is also known as MICL, CLEC12A, CLEC-1 and
25 DCAL2. CLL-1 is a glycoprotein receptor and is expressed in hematopoietic cells. CLL-1 is
absent on uncommitted CD34+/CD38- or CD34+/CD33- stem cells but present on subsets of
CD34+/CD38+ or CD34+/CD33+ progenitor cells (Bakker et al, 2004). In addition, CLL-1 is not
expressed in any other tissue.

CLL-1 expression is seen in acute myeloid leukemia (AML) blasts and leukemic stem
30 cells. CLL-1 is expressed in a variety of leukemias including myelomonocytic leukemia (M4),

acute monocytic leukemia (M5), acute promyelocytic leukemia (M3), chronic myeloid leukemia (CML), chronic myeloproliferative neoplasms and myelodysplastic syndromes (MDS).

CLL-1 is expressed on a subset of leukemic cells related to leukemic stem cells (LSCs), the ablation of which is essential in preventing disease refractoriness and relapse.

5 CD33 (Siglec-3) is a myeloid lineage-specific antigen expressed on early myeloid progenitors, most monocytic cells and approximately 90% of AML blasts, but absent on normal HSCs.

In one aspect of the present disclosure, CD33 antigen is one of the targets for cCAR therapy. CD33 is a transmembrane receptor expressed on 90% of malignant cells in acute
10 myeloid leukemia. Thus, according to the present disclosure, CLL-1 and CD33 target antigens are particularly attractive from a safety standpoint.

In accordance with the present disclosure, the compound CLL-1-CD33 cCARs may be highly effective for therapeutic treatment of chronic myeloid leukemia (CML) population. In chronic myeloid leukemia (CML), there is a rare subset of cells that are CD34+CD38-. This
15 population is considered as comprised of LSCs. Increased number of LSCs is associated with the progression of the disease. A small-molecule Bcr-Abl tyrosine kinase inhibitor (TKI) is shown to significantly improve the overall survival in CP-CML patients. However, LSCs are thought to be resistant to TKI therapy. A novel therapy targeting CML resistant LSCs is urgently needed for treatment of CML and the novel therapy is embodied in the compound CD33CLL-1
20 CAR disclosed in the present disclosure. CLL-1 expression is high in the CD34+CD38- population. In accordance with the present disclosure, the compound CD33CLL-1 CARs is highly effective for therapeutic treatment of this population.

In one embodiment of the present disclosure, leukemic cells expressing both CD33 and CLL-1 in the cCAR are used as a therapeutic treatment. CD33 is expressed on cells of myeloid
25 lineage, myeloid leukemic blasts, and mature monocytes but not normal pluripotent hematopoietic stem cells. CD33 is widely expressed in leukemic cells in CML, myeloproliferative neoplasms, and MDS.

Since a significant number of patients with acute myeloid leukemia (AML) are refractory to standard chemotherapy regimens or experience disease relapse following treatment (Burnett
30 2012), the development of CAR T cell immunotherapy for AML has the potential to address a great clinical need. In the majority of these patients, leukemic cells express both CLL-1 and

CD33, giving broad clinical applicability to the compound CLL-1-CD33 cCAR disclosed herein. Thus, the present disclosure discloses a novel multiple cCAR T/NK cell construct comprising multiple CARs targeting multiple leukemia-associated antigens, thereby offsetting antigen escape mechanism, targeting leukemia cells, including leukemic stem cells, by synergistic effects of co-stimulatory domain activation, thereby providing a more potent, safe and effective therapy.

In further embodiments, the present disclosure provides a method of eradicating or killing leukemic stem cells (LSCs) or bulk leukemic cells expressing CLL-1 or CD33, or both. In this embodiment, a T or NK engineered cell having a CD33 unit and a CLL-1 unit is administered to a patient in need thereof.

In further embodiments, a compound CAR in a T or NK cell may be used to eradicate or kill CD34+ CD38- leukemic stem cells or bulk leukemic cells expressing CLL-1 or CD33 or both.

The present disclosure further discloses a compound CAR construct with enhanced potency of anti-tumor activity against cells co-expressing target antigens, and yet retains sensitivity to tumor cells only expressing one antigen. In addition, each CAR of the compound CAR includes one or two co-stimulatory domains and exhibits potent killing capability in the presence of the specific target.

In this disclosure, it is surprisingly found that CLL-1-CD33 cCAR T-cells are able to eliminate regular leukemic cells and leukemic precursor cells to reduce the risk of relapse, and enhance anti-tumor activities.

In this disclosure, it is also surprisingly found that CLL-1-CD33 cCAR T-cells exhibit a more complete elimination of cancerous cells to reduce antigen escape by hitting hard with multiple targets simultaneously before resistance develops.

In one embodiment, CLL-1-CD33 cCAR T-cell therapy could be developed as a “bridge to transplant”, a supplement to chemotherapy, or a checkpoint blockage (including, but not limited to PD-L1, CTLA-4 inhibitor) or as a standalone therapy for patients with diseases including, but not limited to, acute myeloid leukemia, myelodysplastic syndromes, chronic myeloid leukemia and chronic myeloproliferative disorders.

In another embodiment, CLL-1-CD33cCAR T-cell therapy can use to bring down disease burden to the lowest possible level prior to transplant or thoroughly eliminate MRD, it can be

expected that the relapse rate will decrease and long-term disease-free survival rate will increase, and patient outcomes will be dramatically improved.

In one embodiment, CLL-1-CD33 cCAR T-cell therapy can have further applications for patients with CLL-1+ and/or CD33+ leukemic patients beyond a bridge to bone marrow
5 transplantation. CLL-1-CD33cCAR T-cell therapy as a standalone therapy, or as a part of a patient-individualized immuno-chemotherapy regimen. For elderly patients, or for those with comorbidities who cannot tolerate highly intensive chemotherapy or BMT, this might be a promising strategy to prolong patient's survival time and reserve better life quality.

Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-
10 15sushi or IL-15/IL-15sushi anchor or 4-1BBLwith CLL-1-CD33 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

Without wishing to be bound by theory, it is believed that co-expression of IL-21 or IL-
15 21 anchor with CLL-1-CD33 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a CLL1-CD33 cCAR polypeptide and IL-15/IL-15sushi (SEQ ID NO.60), and corresponding nucleotides (SEQ ID NO. 61).

In one embodiment, the engineered cell includes a CLL-1 CAR polypeptide, 4-1BBL and
20 IL-15/IL-15sushi (SEQ ID NO. 44), and corresponding nucleotides (SEQ ID NO. 45).

In one embodiment, the engineered cell includes a CD33 CAR polypeptide, 4-1BBL and IL-15/IL-15sushi (SEQ ID NO.20), and corresponding nucleotides (SEQ ID NO. 21).

CD123-NKG2D cCAR or CLL-1-NKG2D cCAR or CD33-NKG2D cCAR or BCMA- 25 NKG2D cCAR

NKG2D (NKG2D receptor) is considered a transmembrane protein belonging to the CD94/NKG2 family of C-type lectin-like receptors. NKG2D can bind to at least 8 different ligands that are naturally expressed in AML, multiple myeloma or other leukemias. NKG2D
ligands are induced-self proteins which are virtually absent or present only at very low levels on
30 surface of normal cells but are overexpressed in cancer cells, including AML and multiple myeloma. Therefore, they are good candidates for CAR targeting.

A cCAR contains two units of CARs, a CD123 CAR and NKG2D CAR that target tumor cells expressing CD123 and NKG2D ligands, respectively.

A cCAR contains two units of CARs, a CLL-1 CAR and NKG2D CAR that target tumor cells expressing CLL-1 and NKG2D ligands, respectively.

5 CD123-NKG2D cCAR or CLL-1-NKG2D cCAR or CD33-NKG2D cCAR are able to eliminate leukemias including AML, MDS, CML, and MPN.

In the present disclosure, BCMA-NKG2D cCAR is able to eliminate multiple myeloma.

In this disclosure, the addition of NKG2D as a target to the CD123 CAR or CLL-1 CAR or CD33 CAR enhances the anti-tumor response and reduces the risk of antigen escape associated
10 with disease relapse because NKG2D is widely expressed on AML, MDS, CML and MPN.

BCMA and NKG2D ligands are both widely expressed on multiple myeloma cells, and this high expression allows the BCMA-NKG2D cCAR to have a comprehensive coverage of all potentially cancerous cells. This allows for a more complete elimination of cancerous cells to reduce antigen escape by hitting hard with multiple targets simultaneously before resistance
15 develops.

BCMA-CD38 compound CAR (BCMA-CD38 cCAR)

Current CAR technology efforts in multiple myeloma involve the use of a BCMA (CD269) targeted CART-cell against bulk disease spearheaded by James Kochenderfer (NIH).
20 Those patients in remission after BCMA CAR treatment eventually relapse and this may due to the fact that some myeloma cells are dim (weak) or negative expression for BCMA. Therefore, a single target for CAR based treatment may not be sufficient to prevent myeloma relapse.

CD38 also known as cyclic ADP ribose hydrolase is a glycoprotein is found on the surface of many immune cells including CD4+, CD8+, B lymphocytes, plasma cells, and natural
25 killer cells.

CD38 is another good target for myeloma as its expression is typically high and uniform in myeloma cells and lymphoma cells.

The present disclosure is composed of a single CAR T-cell expressing 2 discrete CAR units in a vector with independent signaling domains can be utilized as a novel approach for
30 targeting multiple antigens and potentially avoiding tumor relapse. A compound CAR (cCAR) comprising of a BCMA CAR linked to a CD38 CAR via a self-cleaving P2A peptide and

expressed both functional CAR molecules on the surface of a T cell. This compound cCAR expression is controlled by a strong promoter, SFFV to ensure adequate CAR expression.

In the present disclosure, BCMA-CD38 cCAR T-cell can provide potent and specific anti- tumor activity in controlling myeloma (Figure 37). Targeting multiple myeloma with a compound CAR targeting both BCMA and CD38 in combination is a very strong strategy. This novel approach circumvents the antigen escape (loss of a single antigen) from selection pressure of single CAR treatment due to combinatorial pressure from a compound design.

In this disclosure, the addition of CD38 as a target to the BCMA CAR enhanced the anti-tumor response by eliminating surviving BCMA⁺CD38⁺ myeloma cells to reduce the risk of relapse.

BCMA and CD38 are both widely expressed on multiple myeloma cells, and this high expression allows the BCMA-CD38 cCAR to have a comprehensive coverage of all potentially cancerous cells. This allows for a more complete elimination of cancerous cells to reduce antigen escape by hitting hard with multiple targets simultaneously before resistance develops.

In one embodiment, BCMA-CD38 directed BCMA-CD38 cCAR therapy is as a “bridge” to bone marrow transplant (BMT) or combination with a heavy chemotherapy plus BMT. BCMA-CD38 cCAR can offer a path to a potentially curative BMT option to many patients that previously would have a residual disease. Current literature supports the idea that reducing the minimal residual disease burden (MRD) to an undetectable level could be associated with improved patient outcomes. This could be extremely beneficial in terms of prevention of relapse for the difficult to treat and highly aggressive malignancies.

In another embodiment, BCMA-CD38 cCAR therapy is able to bring down disease burden to the lowest possible level prior to transplant or thoroughly eliminate MRD, it can be expected that the relapse rate will decrease and long-term disease-free survival rate will increase, and patient outcomes will be dramatically improved.

In one embodiment, BCMA-CD38 cCAR therapy can have further applications for patients with BCMA⁺ and/or CD38⁺ multiple myelomas beyond a bridge to bone marrow transplantation. BCMA-CD38 cCAR therapy as a standalone therapy, or as a part of a patient-individualized immuno-chemotherapy regimen. For elderly patients, or for those with comorbidities who cannot tolerate highly intensive chemotherapy or BMT, this might be a promising strategy to prolong patient’s survival time and reserve better life quality.

In some embodiments, the present disclosure provides a compound CAR polypeptide engineered cell that targets cells expressing BCMA or CD38 antigens or both. The targeted cells may be cancer cells, such as, but not limited to, lymphomas, or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms are selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom's macroglobulinema, heavy chain diseases, solitary bone plamacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma.

It was surprised to find that co-expression of IL-15/IL-15sushi or IL-15/IL-15sushi anchor or 4-1BBLwith BCMA-CD38 cCAR provides long-term durable disease remission by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

Without wishing to be bound by theory, it is believed that co-expression of IL-21 or IL-21 anchor with BCMA-CD38 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

In one embodiment, the engineered cell includes a BCMA-CD38 cCAR polypeptide, 4-1BBL and IL-15/IL-15sushi (SEQ ID NO.40), and corresponding nucleotides (SEQ ID NO. 41).

Without wishing to be bound by theory, it is believed that BCMA-CD38 compound CAR engineered cells provide a better therapeutic outcome in patients suffering from an autoimmune disorder or organ rejection by depletion of B-cells and plasma cells associated with autoimmune disorders.

In some embodiments, a compound CAR (BCMA-CD38 cCAR) targets cells expressing BCMA or CD38 antigens or both. The targeted cells may be cancer cells, such as, without limiting, lymphomas, or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms is selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom's macroglobulinema, heavy chain diseases, solitary bone plasmacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma.

BCMA-CD38 cCAR targeted cells are B cells, immature B cells, memory B cells, plasmablasts, long lived plasma cells, or plasma cells in patients with autoimmune diseases. The

autoimmune diseases include systemic scleroderma, multiple sclerosis, psoriasis, dermatitis, inflammatory bowel diseases (such as Crohn's disease and ulcerative colitis), systemic lupus erythematosus, vasculitis, rheumatoid arthritis, Sjorgen's syndrome, polymyositis, pulmonary alveolar proteinosis, granulomatosis and vasculitis, Addison's disease, antigen-antibody complex mediated diseases, and anti-glomerular basement membrane disease.

In another embodiment, the present disclosure provides a method of treating an autoimmune disease. An autoimmune disorder is selected from a group of diseases including autoimmune disease comprises systemic lupus erythematosus (SLE), multiple sclerosis (MS), Inflammatory bowel disease (IBD), Rheumatoid arthritis, Sjögren syndrome, dermatomyositis, autoimmune hemolytic anemia, Neuromyelitis optica (NMO), NMO Spectrum Disorder (NMOSD), idiopathic thrombocytopenic purpura (ITP), antineutrophil cytoplasmic autoantibodies (ANCA) associated with systemic autoimmune small vessel vasculitis syndromes or microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA, Wegener's granulomatosis, pemphigus vulgaris (PV) and pemphigus foliaceus (PF). Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are chronic and life-threatening blistering diseases caused by autoantibodies.

Compound CD123-CLL-1

Unlike B-cell and plasma cell malignancies, AML is uniquely challenging to treat due to the role of leukemic stem cells (LSCs). LSCs are a population of cells expressing markers of hematopoietic stem cells (CD34+CD38-) that are capable of initiating and maintaining hematopoietic malignancy, producing clonal cell populations that overtake healthy bone marrow. Since LSCs remain mostly in the quiescent phase of the cell cycle, chemotherapy directed against rapidly dividing tumor populations leaves LSCs untouched. Most often it is this elusive population that comprises minimal residual disease (MRD) and is responsible for inevitable relapse after AML treatment. The successful translation of CAR therapy to AML to completely eliminate disease and ensure no relapse occurs will require careful antigen selection to enable the eradication of not just bulk leukemic disease, but also leukemic stem cells.

Single-CAR therapy has recently made breakthroughs in achieving high remission rates in the treatment of previously refractory and relapsed B cell malignancies. Conversely, new treatment approaches for AML are lacking, and CAR therapy offers a beacon of hope. In particular, the application of a compound CAR therapy to AML has the potential to transform its
5 treatment entirely.

CD123 and C-type lectin-like molecule-1 (CLL-1) are present on AML CD34+ CD38- cells in the majority of AML patients. Without wishing to be bound by theory, it is believed that a compound CAR presents the idea in which a single T-cell encoding two discrete CAR units can
10 simultaneously and more broadly target and eradicate LSCs, preventing disease relapse.

The present disclosure is composed of a single CAR T-cell expressing two discrete CAR units in a vector with independent signaling domains that can be utilized as a novel approach for targeting multiple antigens and potentially avoiding tumor relapse. A compound CAR (cCAR) is
15 comprised of a CD123 CAR linked to a CLL-1 CAR via a self-cleaving P2A peptide and expressed both functional CAR molecules on the surface of a T cell.

In one embodiment, CD123-CLL-1 cCAR T-cell therapy could be developed as a “bridge to transplant”, a supplement to chemotherapy, or a checkpoint blockage (including, but not
20 limited to PD-L1, CTLA-4 inhibitor) or as a standalone therapy for patients with diseases including, but not limited to: acute myeloid leukemia, myelodysplastic syndromes, chronic myeloid leukemia and chronic myeloproliferative disorders.

In another embodiment, CD123-CLL-1 cCAR T-cell therapy can be used to thoroughly
25 eliminate MRD. It can be expected that the relapse rate will decrease and long-term disease-free survival rate will increase, and patient outcomes will be dramatically improved.

In one embodiment, CD123-CLL1 cCAR T-cell therapy can have further applications for patients with CD123+ and/or CLL-1+ leukemic patients beyond a bridge to bone marrow transplantation. CD123-CLL-1 cCAR T-cell therapy can be used as a standalone therapy or as a
30 part of a patient-individualized immuno-chemotherapy regimen. For elderly patients or for those

with comorbidities who cannot tolerate highly intensive chemotherapy or BMT, this might be a promising strategy to prolong patients' survival time and reserve a better quality of life.

Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi or IL-15/IL-15sushi anchor or 4-1BBL with CD123-CDLL-1 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

Without wishing to be bound by theory, it is believed that co-expression of IL-21 or IL-21 anchor with CD123-CLL-1 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or by recruiting innate immune cells to cancer cells.

Compound CD38 CARs for T cell malignancies

The present disclosure is composed of a single T-cell expressing two discrete CAR units in a vector with independent signaling domains that can be utilized as a novel approach for targeting multiple antigens and potentially avoiding tumor relapse. A CD38-based compound CAR (cCAR) is comprised of a CD4 CAR or CD5 CAR or CD3 CAR or CD7 CAR linked to a CD38 CAR via a self-cleaving P2A peptide and expresses both functional CAR molecules on the surface of a T cell.

The present disclosure is composed of a single NK-cell expressing two discrete CAR units in a vector with independent signaling domains that can be utilized as a novel approach for targeting multiple antigens and potentially avoiding tumor relapse. A CD38-based compound CAR (cCAR) is comprised of a CD4 CAR or CD5 CAR or CD3 CAR or CD7 CAR linked to a CD38 CAR via a self-cleaving P2A peptide and expresses both functional CAR molecules on the surface of a T cell.

Without wishing to be bound by theory, it is believed that the CD38-based compound cCAR T or NK -cells are able to eliminate T cell lymphoma/ leukemic cells to reduce the risk of relapse due to the antigen escape and enhance anti-tumor activities.

A CD4-CD38 compound CAR (cCAR) comprising of a CD4 CAR is linked to a CD38 CAR via a self-cleaving P2A peptide and expresses both functional CAR molecules on the surface of a T cell.

A CD5-CD38 compound CAR (cCAR) comprising of a CD5 CAR is linked to a CD38

CAR via a self-cleaving P2A peptide and expresses both functional CAR molecules on the surface of a T cell.

In one embodiment, the engineered cell includes a CD5-CD38 chimeric antigen receptor polypeptide (SEQ ID NO. 18), and corresponding nucleotides (SEQ ID NO. 19).

5

A CD7-CD38 compound CAR (cCAR) comprising of a CD4 CAR is linked to a CD38 CAR via a self-cleaving P2A peptide and expresses both functional CAR molecules on the surface of a T cell.

CD56-CD38 CARs for lymphoma/leukemia

10 CD56 is a glycoprotein and functions as the neural cell adhesion molecule. The antigen is expressed on NK cells. CD56 or CD38 is usually present in most cases of 1) aggressive NK cells leukemia/lymphoma, 2) extranodal NK/T lymphoma (nasal type), hepatoplenic T cell lymphoma, and 4) chronic NK cell lymphocytosis.

15 Like CD38, CD56 is also expressed in non-hematologic cells, such as brain cells. The off-target effects would be severe for a patient administered CD56 or CD38 CAR T cells alone.

Without wishing to be bound by theory, it is believed that compound cCAR T cells bearing two CARs and targeting different antigens have a higher affinity of binding to a cell bearing two antigens targeted by cCAR than that of a cell carrying a single cCAR targeted antigen. As a result, it is believed that the compound CAR T cells have a higher capability of
20 trafficking to the tumor than a single CAR T cells. Thus, applicants surprisingly discovered that there was significantly reduced concern of off-target effects when a compound CAR cell based therapy was used.

CD56 is a glycoprotein and functions as the neural cell adhesion molecule. The antigen is expressed on NK cells. Like CD38, CD56 is also expressed in non-hematologic cells, such as
25 brain cells. The off-target effects would be severe for a patient administered CD56 CAR or CD38 CAR T cells. Thus, the invention disclosure provides a method of generating CD56-CD38 cCAR to reduce concerns of off-target effects associated with using CD56 CAR or CD38 CAR alone.

The present invention is composed of a single T-cell expressing two discrete CAR units
30 in a vector with independent signaling domains that can be utilized as a novel approach for targeting CD56 and CD38 simultaneously and potentially avoiding tumor relapse. A CD56-

CD38 compound CAR (cCAR) bears CD56 CAR linked to a CD38 CAR via a self-cleaving P2A peptide and expresses both functional CAR molecules on the surface of a T cell.

The present invention is composed of a single T-cell expressing two discrete CAR units in a vector with independent signaling domains that can be utilized as a novel approach for targeting CD56 and CD38 simultaneously and potentially avoiding tumor relapse. A CD56-CD38 compound CAR (cCAR) bears CD56 CAR linked to a CD38 CAR via a self-cleaving P2A peptide and expresses both functional CAR molecules on the surface of a NK cell.

CD19-CD38 compound CAR (CD19-CD38 cCAR)

While initial remission rates of approximately 90% are commonly seen in patients with B-ALL using CD19CAR, most patients relapse within a year. The relapse is at least in part due to antigen escape. Thus, more effective CAR T cell treatments to prevent relapse are urgently needed.

CD38 is another good target for lymphomas as its expression is typically high and uniform in lymphoma cells. CD38 is expressed in a variety of lymphomas including chronic lymphocytic lymphoma/small lymphocytic lymphoma, follicular lymphoma, primary effusion lymphoma, diffuse large cell lymphoma, lymphoplasmacytic lymphoma.

The present disclosure is composed of a single CAR T-cell expressing two discrete CAR units in a vector with independent signaling domains can be utilized as a novel approach for targeting multiple antigens and potentially avoiding tumor relapse. A compound CAR (cCAR) comprising of a CD19 CAR linked to a CD38 CAR via a self-cleaving P2A peptide and expressed both functional CAR molecules on the surface of a T cell. This compound cCAR expression is controlled by a strong promoter, SFFV to ensure adequate CAR expression.

In the present disclosure, CD19-CD38 cCAR T-cell can provide potent and specific anti-tumor activity in controlling lymphoma. Targeting multiple myeloma with a compound CAR targeting both BCMA and CD19 in combination is a very strong strategy. This novel approach circumvents the antigen escape (loss of a single antigen) from selection pressure of single CAR treatment due to combinatorial pressure from a compound design.

In this disclosure, the addition of CD38 as a target to the BCMA CAR enhanced the anti-tumor response by eliminating surviving BCMA⁺CD38⁺ lymphomas to reduce the risk of relapse.

CD19 and CD38 are both widely expressed on multiple myeloma cells, and this high expression allows the CD19-CD38 cCAR to have a comprehensive coverage of all potentially lymphoma cells. This allows for a more complete elimination of cancerous cells to reduce antigen escape by hitting hard with multiple targets simultaneously before resistance develops.

5 In one embodiment, CD19-CD38 directed BCMA-CD38 cCAR therapy is as a “bridge” to bone marrow transplant (BMT) or combination with a heavy chemotherapy plus BMT. CD19-CD38 cCAR can offer a path to a potentially curative BMT option to many patients that previously would have a residual disease. Current literature supports the idea that reducing the minimal residual disease burden (MRD) to an undetectable level could be associated with
10 improved patient outcomes. This could be extremely beneficial in terms of prevention of relapse for the difficult to treat and highly aggressive malignancies.

In another embodiment, CD19-CD38 cCAR therapy is able to bring down disease burden to the lowest possible level prior to transplant or thoroughly eliminate MRD, it can be expected that the relapse rate will decrease and long-term disease-free survival rate for lymphoma will
15 increase, and patient outcomes will be dramatically improved.

In one embodiment, CD19-CD38 cCAR therapy can have further applications for patients with CD19+ and/or CD38+ multiple myelomas beyond a bridge to bone marrow transplantation. CD19-CD38 cCAR therapy as a standalone therapy, or as a part of a patient-individualized immuno-chemotherapy regimen. For elderly patients, or for those with comorbidities who
20 cannot tolerate highly intensive chemotherapy or BMT, this might be a promising strategy to prolong patient’s survival time and reserve better life quality.

In some embodiments, the present disclosure provides a compound CAR polypeptide engineered cell that targets cells expressing CD19 or CD38 antigens or both. The targeted cells may be cancer cells, such as, but not limited to, lymphomas. In further embodiments,
25 lymphomas are selected from without limiting, B-ALL, high grade B cell lymphoma, low grade B-cell lymphoma, diffuse large B cell lymphoma, Burkett lymphoma, mantle cell lymphoma, CLL, marginal zone B cell lymphoma and follicular lymphoma.

Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi or IL-15/IL-15sushi anchor or 4-1BBL with CD19-CD38 cCAR provides long-term
30 durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

Without wishing to be bound by theory, it is believed that co-expression of IL-21 or IL-21 anchor with CD19-CD38 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target cancer cells or recruiting innate immune cells to cancer cells.

5 Without wishing to be bound by theory, it is believed that CD19-CD38 compound CAR engineered cells provide a better therapeutic outcome in patients suffering from an autoimmune disorder or organ rejection by depletion of B-cells and plasma cells associated with autoimmune disorders.

10 In some embodiments, a compound CAR (BCMA-CD38 cCAR) targets cells expressing BCMA or CD38 antigens or both. The targeted cells may be cancer cells, such as, without limiting, lymphomas, or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms is selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom's macroglobulinemia, heavy chain diseases, solitary bone plasmacytoma, monoclonal gammopathy of undetermined significance (MGUS) and
15 smoldering multiple myeloma.

BCMA-CD38 cCAR targeted cells are B cells, immature B cells, memory B cells, plasmablasts, long lived plasma cells, or plasma cells in patients with autoimmune diseases. The autoimmune diseases include systemic scleroderma, multiple sclerosis, psoriasis, dermatitis, inflammatory bowel diseases (such as Crohn's disease and ulcerative colitis), systemic lupus
20 erythematosus, vasculitis, rheumatoid arthritis, Sjorgen's syndrome, polymyositis, pulmonary alveolar proteinosis, granulomatosis and vasculitis, Addison's disease, antigen-antibody complex mediated diseases, and anti-glomerular basement membrane disease.

In another embodiment, the present disclosure provides a method of treating an autoimmune disease. An autoimmune disorder is selected from a group of diseases including
25 autoimmune disease comprises systemic lupus erythematosus (SLE), multiple sclerosis (MS), Inflammatory bowel disease (IBD), Rheumatoid arthritis, Sjögren syndrome, dermatomyosities, autoimmune hemolytic anemia, Neuromyelitis optica (NMO), NMO Spectrum Disorder (NMOSD), idiopathic thrombocytopenic purpura (ITP), antineutrophil cytoplasmic autoantibodies (ANCA) associated with systemic autoimmune small vessel vasculitis
30 syndromes or microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), Wegener's granulomatosis, pemphigus vulgaris (PV) and pemphigus foliaceus (PF). Pemphigus

vulgaris (PV) and pemphigus foliaceus (PF) are chronic and life-threatening blistering diseases caused by autoantibodies.

BCMA-CD19 compound CAR (BCMA-CD19 cCAR)

While killing multiple myeloma cells can provide short-term relief, LSCs (myeloma
5 leukemic stem cells), if not destroyed, will always re-grow, causing the patient to relapse. It is imperative that LSCs be destroyed to achieve durable cures for multiple myeloma disease. Without wishing to be bound by theory, it is believed that a small subset of multiple myeloma cells is stem cells that are CD19 positive and associated with disease progression and relapses, and a bulky myeloma cell population is BCMA positive. Therefore, it is critical to develop new
10 therapies that can specifically target both the myeloma stem cell population and the bulky myeloma population. A compound CAR in the present disclosure targets BCMA+ and/or CD19+ positive populations of multiple myeloma cells and is embodied herein.

In some embodiments, the present disclosure provides a method of eradicating or killing myeloma stem cells (LSCs) or bulk myeloma cells expressing CD19 and/ or BCMA. In this
15 embodiment, a T or NK engineered cell having a BCMA unit and a CD19 unit is administered to a patient in need thereof.

In some embodiments, the disclosed disclosure comprises methods and compositions of deleting both BCMA and CD19 populations in multiple myeloma to prevent relapses using a BCMA-CD19 cCAR. CAR is more powerful in eliminating myeloma cells when combination of
20 two units of BCMA and CD19 (BCMA-CD19) together in a vector or a cell.

In further embodiments, a compound CAR, BCMA-CD19 cCAR in a T or NK cell may be used to eradicate or kill BCMA+CD19+ or BCMA+CD19- or BCMA-CD19+ populations.

In some embodiments, the disclosed disclosure comprises methods and compositions of deleting both BCMA and CD19 populations in multiple myeloma to prevent relapses using a
25 BCMA-CD19 cCAR. . CAR is more powerful in eliminating myeloma cells when combination of two units of BCMA and CD19 (BCMA-CD19) together in a vector or a cell.

In some embodiments, CD19+ populations can be early precursors for multiple myeloma cells, and CD19-BCMA+ cells can be more differentiated malignant multiple myeloma cells. In some embodiments, the disclosed invention comprises methods and compositions of deleting
30 both early precursor of multiple myeloma cells and more differential malignant multiple myeloma cells using a BCMA-CD19b cCAR (a version of BCMA-CD19 cCAR) T or NK cell.

In a further embodiment, the disclosed disclosure comprises methods and compositions of targeting both early precursor and more differential malignant cells to completely eliminate malignant clones for multiple myeloma using a BCMA-CD19b cCAR T or NK cell.

5 The present disclosure further discloses a compound CAR construct with enhanced potency of anti-myeloma cell activity against cells co-expressing target antigens, and yet retains sensitivity to tumor cells only expressing one antigen. In addition, each CAR of the compound CAR includes one or two co-stimulatory domains and exhibits potent killing capability in the presence of the specific target.

10 Without wishing to be bound by theory, it is believed that co-expression of IL-15/IL-15sushi or IL-15/IL-15sushi anchor or 4-1BBL with BCMA-CD19 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target myeloma cells or recruiting innate immune cells to myeloma cells.

15 In some embodiments, a compound CAR (BCMA-CD19 cCAR) targets cells expressing BCMA or CD19 antigens or both. The targeted cells may be cancer cells, such as, without limiting, lymphomas, or leukemias or plasma cell neoplasms. In further embodiments, plasma cell neoplasms is selected from plasma cell leukemia, multiple myeloma, plasmacytoma, heavy chain diseases, amyloidosis, waldestrom's macroglobulinema, heavy chain diseases, solitary bone plasmacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma.

20 Without wishing to be bound by theory, it is believed that co-expression of IL-21 or IL-IL-21 anchor with BCMA-CD19 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target myeloma cells or recruiting innate immune cells to myeloma cells.

25 Without wishing to be bound by theory, it is believed that co-expression of IL-18 or IL-IL-18 anchor with BCMA-CD19 cCAR provides long-term durable remission in patients by increasing the sensitivity of CAR recognition of target myeloma cells or recruiting innate immune cells to myeloma cells.

30 In some embodiments, the disclosure provides a method of depleting B cells, immature B cells, memory B cells, plasmablasts, long lived plasma cells, or plasma cells in patients with an autoimmune disease by administering to patients CAR or compound CAR (BCMA-CD19 cCAR) T cells or NK cells.

BCMA-CD19 cCAR targeted cells are B cells, immature B cells, memory B cells, plasmablasts, long lived plasma cells, or plasma cells in patients with autoimmune diseases. The autoimmune diseases include systemic scleroderma, multiple sclerosis, psoriasis, dermatitis, inflammatory bowel diseases (such as Crohn's disease and ulcerative colitis), systemic lupus erythematosus, vasculitis, rheumatoid arthritis, Sjorgen's syndrome, polymyositis, pulmonary alveolar proteinosis, granulomatosis and vasculitis, Addison's disease, antigen-antibody complex mediated diseases, and anti-glomerular basement membrane disease.

In some embodiments, immune cells including B cells, immature B cells, memory B cells, plasmablasts, long lived plasma cells, or plasma cells in patients with autoimmune diseases can be eliminated by a BCMA and CD19 bispecific CAR T cell or bispecific antibody.

In another embodiment, the present disclosure provides a method of treating an autoimmune disease. An autoimmune disorder is selected from a group of diseases including autoimmune disease comprises systemic lupus erythematosus (SLE), multiple sclerosis (MS), Inflammatory bowel disease (IBD), Rheumatoid arthritis, Sjögren syndrome, dermatomyositis, autoimmune hemolytic anemia, Neuromyelitis optica (NMO), NMO Spectrum Disorder (NMOSD), idiopathic thrombocytopenic purpura (ITP), antineutrophil cytoplasmic autoantibodies (ANCA) associated with systemic autoimmune small vessel vasculitis syndromes or microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA, Wegener's granulomatosis, Pemphigus vulgaris (PV) and pemphigus foliaceus (PF).

An organ transplant represents a new life for a person and organs that can be transplanted could include the kidneys, heart, lungs, pancreas and intestine. However, many patients are unable to receive a potentially life-saving organ because of pre-existing or developing donor-specific antibody against the donor's antigens such human leukocyte antigens (HLA). Thus, patients may lose the donated organ. Currently there are few treatment options available for antibody mediated rejection, and an enormous unmet need in the field for efficacious treatment of antibody mediated rejection. Deletion of B cells or plasma cells or both using CAR T/NK cell provide a therapy for antibody-mediated rejection.

BCMA-CD19 cCAR or CD19-CD38 cCAR or BCMA-CD38 cCAR targeted cells are B cells, immature B cells, memory B cells, plasmablasts, long lived plasma cells, or plasma cells in patients with the antibody-mediated rejection associated with organ rejections.

Engineered cell having CAR polypeptide and enhancer

In another embodiment, the present disclosure provides an engineered cell having at least one chimeric antigen receptor polypeptide and an enhancer.

In another embodiment, the present disclosure provides an engineered cell having at least one chimeric antigen receptor polypeptide and at least one enhancer.

5 In one embodiment, the present disclosure provides an engineered cell having at least two distinct chimeric antigen receptor polypeptides and an enhancer.

In one embodiment, the present disclosure provides an engineered cell having at least two distinct chimeric antigen receptor polypeptides and at least one enhancer.

10 As used herein, an enhancer includes a biological molecule that promotes or enhances the activity of the engineered cell having the chimeric antigen receptor polypeptide. Enhancers include cytokines. In another embodiment, enhancers include IL-2, IL-7, IL-12, IL-15, IL-18, IL-21, IL-21 anchor, PD-1, PD-L1, CSF1R, CTAL-4, TIM-3, and TGFR beta, receptors for the same, and functional fragments thereof.

15 Enhancers may be expressed by the engineered cell described herein and displayed on the surface of the engineered cell or the enhancer may be secreted into the surrounding extracellular space by the engineered cell. Methods of surface display and secretion are well known in the art. For example, the enhancer may be a fusion protein with a peptide that provides surface display or secretion into the extracellular space.

20 The effect of the enhancer may be complemented by additional factors such as enhancer receptors and functional fragments thereof. The additional factors may be co-expressed with the enhancer as a fusion protein, or expressed as a separate polypeptide and secreted into the extracellular space.

25 Enhancers can be cytokines secreted from engineered CAR cells and are designed to co-express with the CAR polypeptide. A massive release occurs upon CAR engagement of cognate antigen. Inflammatory cells surrounding tumor cells have a significant correlation with cancer cell progression and metastasis. Inflammatory cells could include T cells and innate immune response cells, such as NK cells, macrophages, and dendritic cells and their proliferation and anti-tumor activity are regulated by cytokines. CAR cells such as CAR T or NK cells bind to targeted cancer cells and trigger massive secretion of enhancers from the expansion of CAR
30 T/NK cells. The secreted enhancers efficiently promote survival, differentiation and activation of immune response cells against cancer cells. The co-expression of an enhancer(s) with CAR

can supplement the defect that CAR T or NK cells are unable to eliminate non-targeting cancer cells

CAR cells can be a carrier of cytokines, and cytokines can be delivered to targeted cancer sites by CAR cells to reduce systemic toxicity with high-dose exogenous cytokines.

5 To improve sustained survival or long-lived persistence of CAR cells, a membrane bound enhancer (s) can be co-expressed with CAR to improve CAR persistency

In one embodiment, the enhancer is IL-15. In this instance, the additional factor described above is the IL-15 receptor, and functional fragments thereof. Functional fragments include the IL-15 receptor, IL-15RA, and the sushi domain of IL-15RA (IL-15sushi). Soluble
10 IL-15RA or IL15sushi profoundly potentiates IL-15 functional activity by prevention of IL-15 degradation. Soluble IL-15/IL-15RA or IL-15/IL-15sushi complexes are stable and much more stimulatory than IL-15 alone in vivo.

In one embodiment, IL-15 is co-expressed as a fusion protein with at least one of IL-15 receptor, IL-15RA, and the sushi domain of IL-15RA (IL-15sushi). In one embodiment, the IL-
15 15 receptor, IL-15RA, or the sushi domain of IL-15RA (IL-15sushi) is at the N-terminus of IL-15. In another embodiment, , the IL-15 receptor, IL-15RA, or the sushi domain of IL-15RA (IL-15sushi) is at the C-terminus of IL-15. As used herein, IL-15/IL-15 sushi denotes that IL-15 sushi is at the C-terminus of IL-15 in a fusion protein; and IL-15sushi/il-15 denotes that IL-15 sushi is at the N-terminus of IL-15 in a fusion protein.

20 In some embodiments, IL-15 and the IL-15 receptor or functional fragments thereof polypeptide is on a single polypeptide molecule and is separated by a peptide linker, the peptide linker may be 1-25 amino acid residues in length, 25-100 amino acid residues in length, or 50-200 amino acid residues in length. This linker may include a high efficiency cleavage site described herein.

25 Interleukin (IL)-15 and its specific receptor chain, IL-15R α (IL-15-RA) play a key functional role in various effector cells, including NK and CD8 T cells. CD8+ T cells can be modified to express autocrine growth factors including, but not limited to, IL-2, IL-7, IL-21 or IL-15, to sustain survival following transfer in vivo. Without wishing to be bound by theory, it is believed that IL-15 overcomes the CD4 deficiency to induce primary and recall memory CD8T
30 cells. Overexpression of IL-15-RA or an IL-15 IL-RA fusion on CD8 T cells significantly enhances its survival and proliferation in-vitro and in-vivo. In some embodiments, CD4 CAR or

CD3 CAR or CD5 CAR or CD20 CAR, CD33 CAR, CLL-1 or CD123 CAR, CD19 CAR or CD45 CAR or GD2 CAR, BCMA CAR or any CAR is co-expressed with at least one of IL-15, IL15RA and IL-15/IL-15RA or IL15-RA/IL-15 or IL-15/IL-15sushi, or a part or a combination thereof, to enhance survival or proliferation of CAR T or NK, and to improve expansion of memory CAR CD8+ T cells or NK cells.

CD4 CAR or CD7 CAR, CD3 CAR or CD5 CAR or CD20 CAR, CD33 CAR, CLL-1 or CD123 CAR, CD19 CAR or GD2 CAR or CD45 CAR or BCMA CAR or any CARs co-expressed with at least one of IL-15/IL-15sushi or a part or a combination thereof, to enhance survival or proliferation of CAR NK, and to improve expansion of memory CAR CD8+ T cells.

It is surprisingly found that CAR co-expression of IL-15/IL-15sushi is important for the longer persistence and enhanced activity of the T cells and NK cells targeting tumor cells. It is surprisingly found that CAR co-expression of IL-15/IL-15sushi is important for the T cells or NK T cells, and NK cells targeting tumor cells and preventing cancer relapses. It is surprisingly found that CAR NK cells or NK cells can extend survival when co-expressing with IL-15/IL-15sushi.

The present disclosure provides an engineered cell having a CAR polypeptide as described herein and at least one of IL-15, IL-15RA, IL-15sushi, IL-15/IL-15RA, IL-15-RA/IL-15, IL-15/IL-15sushi, IL15sushi/IL-15, fragment thereof, a combination thereof, to enhance survival or persistence or proliferation of CAR T or NK T or NK cells for treating cancer in a patient.

In one embodiment, the engineered cell includes a CD5 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO. 48), and corresponding nucleotides (SEQ ID NO. 49).

In one embodiment, the engineered cell includes a CD4 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO. 22), and corresponding nucleotides (SEQ ID NO. 23).

In one embodiment, the engineered cell includes a CD4 chimeric antigen receptor polypeptide, 4-1BBL and IL-15/IL-15sushi (SEQ ID NO. 20), and corresponding nucleotides (SEQ ID NO. 21).

In one embodiment, the engineered cell includes a CD3 chimeric antigen receptor polypeptide, 4-1BBL and IL-15/IL-15sushi (SEQ ID NO. 18), and corresponding nucleotides (SEQ ID NO. 19).

5 In one embodiment, the engineered cell includes a CD19 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO. 24), and corresponding nucleotides (SEQ ID NO. 25).

In one embodiment, the engineered cell includes a CD19 chimeric antigen receptor polypeptide, 4-1BBL and IL-15/IL-15sushi (SEQ ID NO. 26), and corresponding nucleotides (SEQ ID NO. 27).

10 In one embodiment, the engineered cell includes a CD33 chimeric antigen receptor polypeptide, 4-1BBL and IL-15/IL-15sushi (SEQ ID NO. 30), and corresponding nucleotides (SEQ ID NO. 31).

In one embodiment, the engineered cell includes a CD123 chimeric antigen receptor polypeptide, 4-1BBL and IL-15/IL-15sushi (SEQ ID NO. 32), and corresponding nucleotides
15 (SEQ ID NO. 33).

In one embodiment, the engineered cell includes a BCMA chimeric antigen receptor polypeptide, 4-1BBL and IL-15/IL-15sushi (SEQ ID NO. 38), and corresponding nucleotides (SEQ ID NO. 39).

In one embodiment, the engineered cell includes a GD2 chimeric antigen receptor
20 polypeptide, 4-1BBL and IL-15/IL-15sushi (SEQ ID NO. 46), and corresponding nucleotides (SEQ ID NO. 47).

In one embodiment, the engineered cell includes a GD2 chimeric antigen receptor polypeptide (SEQ ID NO. 56), and corresponding nucleotides (SEQ ID NO. 57).

25 In one embodiment, the engineered cell includes a GD2 chimeric antigen receptor polypeptide and 4-1BBL (SEQ ID NO. 56), and corresponding nucleotides (SEQ ID NO. 57).

In one embodiment, the engineered cell includes a CD45 chimeric antigen receptor polypeptide and IL-15/IL-15sushi (SEQ ID NO. 54), and corresponding nucleotides (SEQ ID NO. 55).

30 In another embodiment, the present disclosure provides an engineered cell having at least one of recombinant IL-15, IL-15RA, IL-15sushi, IL-15/IL-15RA, IL15-RA/IL-15, IL-15/IL-15sushi, IL15sushi/IL-15, functional fragment thereof, and combination thereof; and at least one

distinct CAR polypeptide wherein the antigen recognition domain includes GD2, GD3, interleukin 6 receptor, ROR1, PSMA, PSCA (prostate stem cell antigen), MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE-6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, MUC1, MUC2, MUC3, MUC4, MUC5, CD30, EGFRvIII, CD33, CD123, CLL-1, immunoglobulin kappa and lambda, CD38, CD52, CD19, CD20, CD22, CD38, BCMA, CS1, BAFF receptor, TACI, CD3, CD4, CD8, CD5, CD7, CD2, and CD138.

Without wishing to be bound by theory, it is believed that IL-15/IL-15sushi and other types of IL-15 or IL-15RA proteins or protein fragments thereof provide synergistic efficacy of a CAR polypeptide when combined with checkpoint inhibitors or modulators (e.g. anti-PD-1).

In one embodiment, the present disclosure provides a method of providing long-term durable remission in patients suffering from cancer by administering a CAR engineered cell that co-expresses IL-21 or IL-12 anchor to a patient in need thereof (Figure 24 and 25). Without wishing to be bound by theory, it is believed that co-expression of IL-21 or IL-21 anchor with a CAR provides long-term durable remission in patients by increasing the persistence of CAR engineered cells.

Without wishing to be bound by theory, it is also believed that co-expression of secreting IL-21 with a CAR polypeptide provides long-term durable remission in patients by affecting tumor micro-environment resulting in reduction of immunosuppression and promotion of innate cell proliferation or functions.

Without wishing to be bound by theory, it is believed that CAR co-expression of secreting IL-21 or IL-21 anchor is important for the longer persistence and enhanced activity of the T cells, NK T cells and NK cells targeting tumor cells. CAR NK cells or NK cells or NK T cells can extend survival when co-expressing with IL-21 or IL-21 anchor.

In one embodiment, the present disclosure provides a method related to that CAR T or NK cells targeting tumor cells can be a carrier to delivery an enhancer, IL-21 to the tumor micro-environment. CAR T or NK cells are engineered to co-express a secretory IL-21. Engineered CAR T or NK T cells or NK cells in tumor microenvironment, target tumor cells, binding to the CAR targeting antigen, and triggering lysis of tumor cells and massive secretion of soluble IL-21 from the expansion of CAR T or NK T cells or NK cells.

In particular embodiments, elimination of tumor can be achieved by combination of at

least one or more of the following steps:

- (1) binding of an CAR engineered T cell or NK cell or NK T cell disclosed herein to a portion of tumor cells by targeting CAR antigen(s);
- (2) Triggering of a massive secretion of IL-21 from expansion of CAR T/NK cells, which co-
5 express this molecule;
- (3) Recruiting and stimulating a variety of innate and adaptive immune cells against tumor;
- (4) Reducing tumor suppression that is present in tumor by administration of a checkpoint blockage such as PD-L1 and CTLA-4 inhibitor.

Without wishing to be bound by theory, it is believed that the combination of steps
10 described above provide potent anti-tumor effects via a concerted innate and adaptive immune response.

In another embodiment, the present disclosure provides an engineered cell having IL-21 or IL-21 anchor, functional fragment thereof, and combination thereof; and at least one distinct CAR polypeptide wherein the antigen recognition domain includes GD2, GD3, interleukin 6
15 receptor, ROR1, PSMA, PSCA (prostate stem cell antigen), MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE- 6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, MUC1, MUC2, MUC3, MUC4, MUC5, CD30, EGFRvIII, CD33, CD123, CLL-1, immunoglobulin kappa and lambda, CD38, CD52, CD19, CD20, CD22, CD38, BCMA, CS1, BAFF receptor, TACI, CD3, CD4,
20 CD8, CD5, CD7, CD2, and CD138.

In one embodiment, the present disclosure provides a method of providing long-term durable remission in patients suffering from cancer by administering a CAR engineered cell that co-expresses IL-18 or IL-18 anchor to a patient in need thereof (Figure 26 and 27). Without
25 wishing to be bound by theory, it is believed that co-expression of IL-18 or IL-18 anchor with a CAR provides long-term durable remission in patients by increasing the persistence of CAR engineered cells.

Without wishing to be bound by theory, it is also believed that co-expression of secreting IL-18 with a CAR polypeptide provides long-term durable remission in patients by affecting tumor micro-environment resulting in reduction of immunosuppression and promotion of innate
30 cell proliferation or functions.

Without wishing to be bound by theory, it is believed that CAR co-expression of

secreting IL-18 or IL-18 anchor is important for the longer persistence and enhanced activity of the T cells and NK cells targeting tumor cells. CAR NK cells or NK cells can extend survival when co-expressing with IL-18 or IL-18 anchor.

In one embodiment, the present disclosure provides a method related to that CAR T or NK cells targeting tumor cells can be a carrier to delivery an enhancer, IL-18 to the tumor micro-environment. CAR T or NK cells are engineered to co-express a secretory IL-18. Engineered CAR T or NK cells in tumor microenvironment, target tumor cells, binding to the CAR targeting antigen, and triggering lysis of tumor cells and massive secretion of soluble IL-18 from the expansion of CAR T or NK cells.

In particular embodiments, elimination of tumor can be achieved by combination of at least one or more of the following steps:

- (1) binding of an CAR engineered T cell or NK cell disclosed herein to a portion of tumor cells by targeting CAR antigen(s);
- (2) Triggering of a massive secretion of IL-18 from expansion of CAR T/NK cells, which co-express this molecule;
- (3) Recruiting and stimulating a variety of innate and adaptive immune cells against tumor;
- (4) Reducing tumor suppression that is present in tumor by administration of a checkpoint blockage such as PD-L1 and CTLA-4 inhibitor.

Without wishing to be bound by theory, it is believed that the combination of steps described above provide potent anti-tumor effects via a concerted innate and adaptive immune response.

In another embodiment, the present disclosure provides an engineered cell having IL-18 or IL-18 anchor, functional fragment thereof, and combination thereof; and at least one distinct CAR polypeptide wherein the antigen recognition domain includes GD2, GD3, interleukin 6 receptor, ROR1, PSMA, PSCA (prostate stem cell antigen), MAGE A3, Glycolipid, glypican 3, F77, GD-2, WT1, CEA, HER-2/neu, MAGE-3, MAGE-4, MAGE-5, MAGE-6, alpha-fetoprotein, CA 19-9, CA 72-4, NY-ESO, FAP, ErbB, c-Met, MART-1, MUC1, MUC2, MUC3, MUC4, MUC5, CD30, EGFRvIII, CD33, CD123, CLL-1, immunoglobulin kappa and lambda, CD38, CD52, CD19, CD20, CD22, CD38, BCMA, CS1, BAFF receptor, TACI, CD3, CD4, CD8, CD5, CD7, CD2, and CD138.

In some embodiments, targeting more than one different antigen can be achieved by

pooled CAR engineered cells, which are generated by at least two separate CAR T or NK cells. As used herein, pooled CAR engineered cells include a population of engineered cells having more than one distinct CAR polypeptide unit. By way of example, pooled engineered cells include a population of engineered cells with a distinct CAR polypeptide and a population of
5 engineered cells with a different and distinct CAR polypeptide. Furthermore, the pooled CAR engineered cells include engineered cells having cCAR polypeptides.

Methods of generating engineered cells

Any of the polynucleotides disclosed herein may be introduced into an engineered cell by any method known in the art.

10 In one embodiment, CAR polynucleotides are delivered to the engineered cell by any viral vector as disclosed herein.

In one embodiment, to achieve enhanced safety profile or therapeutic index, the any of the engineered cells disclosed herein be constructed as a transient RNA-modified “biodegradable” version or derivatives, or a combination thereof. The RNA-modified CARs of
15 the present disclosure may be electroporated into T cells or NK cells. The expression of the compound CAR may be gradually diminished over few days.

In some embodiments of the present disclosure, any of the engineered cells disclosed herein may be constructed in a transposon system (also called a “Sleeping Beauty”), which integrates the CAR DNA into the host genome without a viral vector.

20 In some embodiments of the present disclosure, any of the engineered cells disclosed herein may be introduced by two vectors, and each vector bears a unit of CAR or an enhancer.

Methods of generating an engineered cell having multiple CAR units

In another embodiment, the present disclosure provides a method making an engineered cell having at least two CAR units.

25 In some embodiments, multiple units of CAR are expressed in a T or NK cell using bicistronic or multicistronic expression vectors. There are several strategies which can be employed to construct bicistronic or multicistronic vectors including, but not limited to, (1) multiple promoters fused to the CARs’ open reading frames;(2) insertion of splicing signals between units of CAR; fusion of CARs whose expressions are driven by a single promoter;(3)
30 insertion of proteolytic cleavage sites between units of CAR (self-cleavage peptide); and (4)

insertion of internal ribosomal entry sites (IRESs); (5) separate two vectors to express different units of CAR.

In a preferred embodiment, multiple CAR units are expressed in a single open reading frame (ORF), thereby creating a single polypeptide having multiple CAR units. In this
5 embodiment, an amino acid sequence or linker containing a high efficiency cleavage site is disposed between each CAR unit.

As used herein, high cleavage efficiency is defined as more than 50 %, more than 70 %, more than 80%, or more than 90% of the translated protein is cleaved. Cleavage efficiency may be measured by Western Blot analysis, as described by Kim 2011.

10 Furthermore, in a preferred embodiment, there are equal amounts of cleavage product, as shown on a Western Blot analysis.

Examples of high efficiency cleavage sites include porcine teschovirus-1 2A (P2A), FMDV 2A (abbreviated herein as F2A); equine rhinitis A virus (ERAV) 2A (E2A); and Thoseaasigna virus 2A (T2A), cytoplasmic polyhedrosis virus 2A (BmCPV2A) and flacherie
15 Virus 2A (BmIFV2A), or a combination thereof. In a preferred embodiment, the high efficiency cleavage site is P2A. High efficiency cleavage sites are described in Kim JH, Lee S-R, Li L-H, Park H-J, Park J-H, Lee KY, et al. (2011) High Cleavage Efficiency of a 2A Peptide Derived from Porcine Teschovirus-1 in Human Cell Lines, Zebrafish and Mice. PLoS ONE 6(4): e18556, the contents of which are incorporated herein by reference.

20 In embodiments, wherein multiple CAR units are expressed in a single open reading frame (ORF), expression is under the control of a strong promoter. Examples of strong promoters include the SFFV promoter, and derivatives thereof.

When designing longer gene constructs, the level of protein expression drops significantly with each 1 kb of additional length. Therefore, an initial screen of several antigen
25 recognition sequences is preferred to find the combination that yields both the highest transduction efficiency along with highest target cell lysis. Additionally, it is preferred to avoid very high CAR expression which leads to tonic effects and poor lysis caused by single chain aggregation on the cell surface.

In embodiments, wherein multiple CAR units are expressed in a cell, CAR-CAR
30 interaction between the hinge region of each individual CAR is preferred to be avoided. The

interaction site of the hinge is preferred to be excluded or each unit of CARs uses different hinge regions to avoid their interaction.

In some embodiments, wherein multiple CAR units are expressed in a cell, different nucleotide sequences for each domain in common, such as leader sequence, hinge and
5 transmembrane regions, and CD3zeta region, are preferred to avoid homologous recombination, while maintaining the same amino acid sequence.

In some embodiments, wherein multiple CAR units are created, the choice of target antigen is preferred based on which will give the best therapeutic effect, based on medical knowledge and background.

10 In some embodiments, targeting more than one different antigen can be achieved by pooled CAR engineered cells, which are generated by at least two separate CAR T or NK cells.

It is preferred that co-culture lysis experiments be performed on both on-target cell lines, and off-target cell lines using CAR T or NK cells, to test specificity. Additionally, it is preferred that cell lines expressing only one targeted antigen each be used to demonstrate the ability of
15 each component CAR to lyse. To do this, it is preferred that an off-target cell line be made to synthetically express the desired antigen(s).

In some embodiments, targeting more than one different antigen can be achieved by pooled CAR engineered cells, which are generated by at least two separate CAR T or NK cells.

As used herein, pooled CAR engineered cells include a population of engineered cells
20 having more than one distinct CAR polypeptide unit. By way of example, pooled engineered cells include a population of engineered cells with a distinct CAR polypeptide and a population of engineered cells with a different and distinct CAR polypeptide. Furthermore, the pooled CAR engineered cells include engineered cells having cCAR polypeptides.

Engineered cell having CAR polypeptide and enhancer

25 In another embodiment, the present disclosure provides a method making an engineered cell that expresses at least one CAR unit and an enhancer.

In some embodiments, at least one CAR unit and enhancer is expressed in a T or NK cell using bicistronic or multicistronic expression vectors. There are several strategies which can be employed to construct bicistronic or multicistronic vectors including, but not limited to, (1)
30 multiple promoters fused to the CARs' open reading frames;(2) insertion of splicing signals between units of CAR; fusion of CARs whose expressions are driven by a single promoter;(3)

insertion of proteolytic cleavage sites between units of CAR (self-cleavage peptide); and (4) insertion of internal ribosomal entry sites (IRESs).

In some embodiments, at least one CAR and an enhancer (s) expressing in a T cell or NK cell can be achieved by two separate vectors or viruses.

5 In a preferred embodiment, at least one CAR unit and an enhancer are expressed in a single open reading frame (ORF), thereby creating a single polypeptide having at least one CAR unit and an enhancer. In this embodiment, an amino acid sequence or linker containing a high efficiency cleavage site is disposed between each CAR unit and between a CAR unit and enhancer. In this embodiment, the ORF is under the control of a strong promoter. Examples of strong promoters include the SFFV promoter, and derivatives thereof.

10 Furthermore, in a preferred embodiment, there are equal amounts of cleavage product, as shown on a Western Blot analysis.

Combination therapy

15 The compositions and methods of this disclosure can be used to generate a population of CAR T lymphocyte or NK cells that deliver both primary and co-stimulatory signals for use in immunotherapy in the treatment of cancer. In further embodiments, the present invention for clinical aspects are combined with other agents effective in the treatment of hyperproliferative diseases, such as anti-cancer agents. Anti-cancer agents are capable of reduction of tumor burdens in a subject. Anti-cancer agents include chemotherapy, radiotherapy and immunotherapy.

More than 50 % of persons with cancer will undergo surgery of some type. Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed.

25 The compositions and methods described in the present disclosure may be utilized in conjunction with other types of therapy for cancer, such as chemotherapy, surgery, radiation, gene therapy, and so forth.

In accordance with the present disclosure, natural killer (NK) cells represent alternative cytotoxic effectors for CAR driven killing. Unlike T-cells, NK cells do not need pre-activation and constitutively exhibit cytolytic functions. Further expression of cCARs in NK cells allow

NK cells to effectively kill cancers, particularly cancer cells that are resistant to NK cell treatment.

Further, NK cells are known to mediate anti-cancer effects without the risk of inducing graft-versus-host disease (GvHD).

5 The present disclosure may be better understood with reference to the examples, set forth below. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure

10 Administration of any of the engineered cells described herein may be supplemented with the co-administration of a CAR enhancing agent. Examples of CAR enhancing agents include immunomodulatory drugs that enhance CAR activities, such as, but not limited to agents that target immune-checkpoint pathways, inhibitors of colony stimulating factor-1 receptor (CSF1R) for better therapeutic outcomes. Agents that target immune-checkpoint pathways include small
15 molecules, proteins, or antibodies that bind inhibitory immune receptors CTLA-4, PD-1, and PD-L1, and result in CTLA-4 and PD-1/PD-L1 blockades. As used herein, enhancing agent includes enhancer as described above.

 As used herein, “patient” includes mammals. The mammal referred to herein can be any mammal. As used herein, the term “mammal” refers to any mammal, including, but not limited
20 to, mammals of the order Rodentia, such as mice and hamsters, and mammals of the order Logomorpha, such as rabbits. The mammals may be from the order Carnivora, including Felines (cats) and Canines (dogs). The mammals may be from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla, including Equines (horses). The mammals may be of the order Primates, Ceboids, or Simoids (monkeys) or of the order
25 Anthropoids (humans and apes). Preferably, the mammal is a human. A patient includes subject.

 In certain embodiments, the patient is a human 0 to 6 months old, 6 to 12 months old, 1 to 5 years old, 5 to 10 years old, 5 to 12 years old, 10 to 15 years old, 15 to 20 years old, 13 to 19 years old, 20 to 25 years old, 25 to 30 years old, 20 to 65 years old, 30 to 35 years old, 35 to 40 years old, 40 to 45 years old, 45 to 50 years old, 50 to 55 years old, 55 to 60 years old, 60 to 65
30 years old, 65 to 70 years old, 70 to 75 years old, 75 to 80 years old, 80 to 85 years old, 85 to 90 years old, 90 to 95 years old or 95 to 100 years old.

The terms “effective amount” and “therapeutically effective amount” of an engineered cell as used herein mean a sufficient amount of the engineered cell to provide the desired therapeutic or physiological or effect or outcome. Such, an effect or outcome includes reduction or amelioration of the symptoms of cellular disease. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining what an appropriate “effective amount” is. The exact amount required will vary from patient to patient, depending on the species, age and general condition of the patient, mode of administration and the like. Thus, it may not be possible to specify an exact “effective amount”. However, an appropriate “effective amount” in any individual case may be determined by one of ordinary skill in the art using only routine experimentation. Generally, the engineered cell or engineered cells is/are given in an amount and under conditions sufficient to reduce proliferation of target cells.

Following administration of the delivery system for treating, inhibiting, or preventing a cancer, the efficacy of the therapeutic engineered cell can be assessed in various ways well known to the skilled practitioner. For instance, one of ordinary skill in the art will understand that a therapeutic engineered cell delivered in conjunction with the chemo-adjuvant is efficacious in treating or inhibiting a cancer in a patient by observing that the therapeutic engineered cell reduces the cancer cell load or prevents a further increase in cancer cell load. Cancer cell loads can be measured by methods that are known in the art, for example, using polymerase chain reaction assays to detect the presence of certain cancer cell nucleic acids or identification of certain cancer cell markers in the blood using, for example, an antibody assay to detect the presence of the markers in a sample (e.g., but not limited to, blood) from a subject or patient, or by measuring the level of circulating cancer cell antibody levels in the patient.

Throughout this specification, quantities are defined by ranges, and by lower and upper boundaries of ranges. Each lower boundary can be combined with each upper boundary to define a range. The lower and upper boundaries should each be taken as a separate element.

Reference throughout this specification to “one embodiment,” “an embodiment,” “one example,” or “an example” means that a particular feature, structure or characteristic described in connection with the embodiment or example is included in at least one embodiment of the present embodiments. Thus, appearances of the phrases “in one embodiment,” “in an embodiment,” “one example,” or “an example” in various places throughout this specification

are not necessarily all referring to the same embodiment or example. Furthermore, the particular features, structures or characteristics may be combined in any suitable combinations and/or sub-combinations in one or more embodiments or examples. In addition, it is appreciated that the figures provided herewith are for explanation purposes to persons ordinarily skilled in the art and
5 that the drawings are not necessarily drawn to scale.

As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having,” or any other variation thereof, are intended to cover a non-exclusive inclusion. For example, a process, article, or apparatus that comprises a list of elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherent to
10 such process, article, or apparatus.

Further, unless expressly stated to the contrary, “or” refers to an inclusive “or” and not to an exclusive “or”. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

15 Additionally, any examples or illustrations given herein are not to be regarded in any way as restrictions on, limits to, or express definitions of any term or terms with which they are utilized. Instead, these examples or illustrations are to be regarded as being described with respect to one particular embodiment and as being illustrative only. Those of ordinary skill in the art will appreciate that any term or terms with which these examples or illustrations are
20 utilized will encompass other embodiments which may or may not be given therewith or elsewhere in the specification and all such embodiments are intended to be included within the scope of that term or terms. Language designating such nonlimiting examples and illustrations includes, but is not limited to: “for example,” “for instance,” “e.g.,” and “in one embodiment.”

In this specification, groups of various parameters containing multiple members are
25 described. Within a group of parameters, each member may be combined with any one or more of the other members to make additional sub-groups. For example, if the members of a group are a, b, c, d, and e, additional sub-groups specifically contemplated include any one, two, three, or four of the members, e.g., a and c; a, d, and e; b, c, d, and e; etc.

As used herein, a XXXX antigen recognition domain is a polypeptide that is selective for
30 XXXX. “XXXX” denotes the target as discussed herein and above. For example, a CD38 antigen recognition domain is a polypeptide that is specific for CD38.

As used herein, CDXCAR refers to a chimeric antigen receptor having a CDX antigen recognition domain.

EXAMPLES

BCMA-CS1 cCAR targeting plasma cell diseases such as multiple myeloma

5 Generation of BCMA-CS1 cCAR (BC1cCAR) T-cells

The BC1cCAR construct is a 2-unit CAR composed of a complete BCMA-CAR fused to a complete CS1-CAR by a self-cleaving P2A peptide, enabling independent expression of both CAR receptors separately on the T-cell surface (Fig. 1A). Expression assayed by FACS revealed distinct transduced cells (Fig 1B). A leader, a scFv, a hinge domain (H), a transmembrane domain (TM), a co-stimulatory domain (CD28 or 4-1BB) and the intracellular signaling domain CD3 zeta (CD3) are included in each CAR unit. A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the BCMA-CS1 cCAR molecule on the T-cell surface.

15 BC1cCAR T-cells specifically lyse BCMA⁺ and CS1⁺ myeloma cell lines

To assess the cytotoxicity of BC1cCAR T-cells, we conducted co-culture assays against myeloma cell lines: MM1S (BCMA⁺ CS1⁺), RPMI-8226 (BCMA⁺ CS1^{dim}), and U266 (BCMA⁺ CS1^{dim}). FACS analysis of BC1cCAR cytotoxicity in 24 hour co-cultures show virtually complete lysis of MM1S cells (>90%) at all E:T ratios (Fig. 2A). Similar trends were observed against RPMI-8226 and U266 cells in culture (Fig. 2A, 2B), demonstrating effective bulk cytotoxicity against target populations with varying levels of antigen expression (Fig. 2C).

BC1cCAR T-cells specifically target BCMA⁺ and CS1⁺ populations in primary myeloma samples

To further evaluate the BC1cCAR's ability to kill diverse primary myeloma cell types, primary samples were chosen to exhibit a spectrum of target antigen expression (Figure 3). Flow cytometry analysis of the MM10-G sample revealed a mixed tumor with double positive BCMA⁺ CS1⁺ as well as CS1⁺ only population subsets. MM7-G sample showed a complete BCMA⁺ CS1⁺ phenotype while bone marrow aspirate MM11-G exhibited a noisy BCMA^{dim} CS1^{dim} phenotype. BC1cCAR T-cells showed robust (>80%) dose-dependent ablation of the MM7-G primary patient sample (Fig. 4A).

BC1cCAR also showed targeted and specific lysis ability, by significantly ablating both BCMA⁺ CS1⁺ and BCMA⁻ CS1⁺ population subsets in MM10-G co-cultures. At an E:T ratio of 2:1, BC1cCAR T-cells ablated over 60% of the BCMA⁺ CS1⁺ population, and 70% of the CS1⁺ only population with slight dose dependent increases (Fig 4B). BC1cCAR T-cells were also able to demonstrate dose-dependent cytotoxic activity against the MM11-G cells (Fig. 4C). Across the cytotoxicity screening, BC1cCAR T cells exhibited robust anti-tumor activity against both myeloma cell lines and primary tumor cells expressing different combinations of BCMA and CS1 (Fig. 4D)

10 **Functional evaluation of BC1cCAR antigen specific activity**

We established a model that allowed us to test the BC1cCAR scFv functionality independently. A CML cell line, K562, negative for myeloma markers was overexpressed with either CS1 (CS1-K562) or BCMA (BCMA-K562). After confirming independent antigen expression in each cell line (Fig. 5A), we determined BC1cCAR T-cell targeting functionality through co-culture experiments.

In short-term cultures (overnight), BC1cCAR T-cells exhibited cytotoxic activity against BCMA-K562 cells. There were no off-target effects against wild-type K562 cells negative for either antigen (Fig. 5B). Short-term cultures against CS1-K562 cells also showed similar responses against CS1-expressing target cells. In addition, BC1cCAR T-cells appeared to have a stronger cytotoxic effect than a CS1-specific CAR against CS1-K562 cells (Fig. 5B).

Residual tumor populations possessing a non-target antigen may lead to relapse in patients who have undergone treatment using a single-antigen CAR. Thus, to model more clinically relevant mixed antigen-expressing cell populations, we conducted combined co-culture experiments. BCMA-K562 and CS1-K562 cells were mixed in 1:1 ratios in a sustained (48h) culture to assay for residual antigen positive populations. Next, histograms were constructed that represented populations of T-cells and target tumor cells with residual gated target tumor populations marked (Fig. 5C). We found that compared to control T-cells, BCMA-specific CAR and CS1-specific CAR had profound cytotoxic effects against their respective target populations. However, CS1-CAR left a significant residual BCMA⁺ population, whereas BCMA-CAR achieved a high degree of cytotoxicity but left a small CS1⁺ population. In contrast, the BC1cCAR T-cells effectively depleted both target populations (Fig. 5C).

Tumor re-challenge demonstrates sequential killing ability of BC1cCAR T-cells

We next investigated the ability of BC1cCAR T-cells to kill tumor cells in a sequential manner under unfavorable microenvironments caused by cell lysis, debris, and tumor re-challenge. Using the scheme in Fig. 6A, we conducted long-term co-cultures using MM1S cells as a model myeloma tumor and periodically re-challenged BC1cCAR T-cells and single BCMA-CAR and CS1-CAR T-cells with fresh MM1S cells to simulate tumor expansion or relapse. Even without exogenous cytokines, we found that all CAR treatments depleted target antigens after 48 hours, with significant clustering and T-cell proliferation (Fig. 6B). In contrast, control T-cells showed no response or proliferation, and yielded a tumor cell population twice its initial size. After re-challenging all treatment wells with fresh MM1S cells we found that all CARs still retained a high degree of cytotoxicity. By 108 hours, new MM1S cells were virtually depleted by both BCMA-CAR and the BC1cCAR, while the CS1-CAR displayed incomplete killing of the new MM1S cells (Fig. 6C). All CAR-mediated tumor lysis and cytotoxicity stopped after 168 hours, however, BCMA-CAR and BC1cCAR still showed detectable minority T-cell populations while control T-cells and CS1-CAR T-cells were virtually undetectable (data not shown).

BC1cCAR T-cells exhibit significant control and reduction of tumor in vivo

In order to evaluate the in vivo activity of BC1cCAR T-cells, we developed a myeloma mouse model with luciferase-expressing MM1S cells to induce fluorescence visible tumor formation. The BC1cCAR T-cells significantly reduced tumor burden and prolonged survival in MM1S-injected mice when compared to control T-cells. Mice were given a single dose of BC1cCAR or control T-cells and tumor burden assayed by IVIS imaging (Fig. 7A). There was a highly significant difference ($P < 0.0003$) in IVIS measurement of tumor burdens between the control group and the BC1cCAR treatment group from Day 6 onwards (Fig. 7B). CAR injected mice also had significantly more favorable survival outcomes (Fig. 7C).

Mixed antigen population mouse models demonstrate superior tumor burden control by cCAR expressing cells vs single CAR expressing cells

To model heterogeneous cell populations and potential antigen escape, we injected mice with a 4:1 mix of BCMA:CS1-expressing K562 cells and treated on day 3 with 7.5×10^6 of either control, BCMA-CAR, or BC1cCAR T-cells. CS1-CAR T-cells were excluded on the basis

of inferior in vitro efficacy. On day 3, two control mice died as a result of the injection procedure and were excluded from analysis. Tumor burden was visualized by fluorescence (Fig. 8A). At day 10, both CARs exhibited over 50% tumor reduction compared to GFP control, increasing to over 60% by day 12 (Fig 8A – right). By day 10, BC1cCAR outpaced BCMA-CAR in tumor suppression by 6% and this spread increased to 17% by day 12, potentially modeling the inability of BCMA-CAR to lyse residual CS1-K562 cells (20% of tumor injected). Survival outcomes for all CAR T-cell treated mice were significantly improved over the control group. There was also a significant improvement ($p < 0.05$) in survival for the BC1cCAR group versus the BCMA-CAR group (Fig. 8B). While both CARs were efficacious in controlling tumor growth, the BC1cCAR demonstrates more robust control compared to a single target option.

Enhanced T-cell persistency and maintenance of tumor depletion by compound CAR T-cells in independent antigen mouse models

To assay specific BCMA and CS1 antigen-expressing cell depletion and verify compound scFv efficacy, a third mouse model was constructed in which 4 groups consisting of 5 mice each were injected with either BCMA-K562 or CS1-K562 cells, with control and BC1cCAR T-cells administered to each tumor group ($n = 19$ as a result of an early spontaneous mouse death). At times of sacrifice (various: day 30 – 80+), mice whole blood and liver tissues were screened for T-cell and tumor populations. Both hematological tissue types show consistent tumor presence in control groups when compared to cCAR groups (Fig. 9A, 9B, 10A, 10B). Aggregate tissue analysis of averaged tumor cell populations in both tissues show consistent trends of depleted tumor burden in cCAR treated mice groups (Fig. 9B). In both the blood and liver, control T-cells were unable to persist beyond the 30 day mark and exhibited significant tumor burden in both tissue types (Fig 9B, 9C). In contrast, cCAR treated mice showed significant T-cell expansion and persistency compared to control T-cells across all mice even at day 30+ (Fig. 9C), correlating with observed increased anti-tumor activity and supporting overall improved survival.

Structural organization of BCMA-CS1-IL-15/IL-15sushi (CD269-A7D-CS1-hu63-

IL15/IL15sushi)

BCMA-CS1-IL-15/IL-15sushi (Figure 11A) contains two independent units of CARs, CD269-A7D (also called BCMA CAR or anti-CD269 CAR), and CS1 CAR (also called CS1-hu63 CAR or anti-CS1 CAR). BCMA-CS1-IL-15/IL-15sushi CAR is able to secrete IL-15/IL-15sushi. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion can enhance CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers

CAR expression

Activated human peripheral blood T cells were transduced with the lentiviral vector from CD269-A7D-CS1-hu63-IL15/IL15sushi. CAR. Figure 11B shows the transduction efficiency between activated T cells transduced with either control vector, or CD269-A7D-hu63-IL15/IL15sushi CAR vector, as determined by labeling with goat anti-mouse F(Ab')₂ antibody. Activated T cells transduced with the CAR vectors resulted in 23.7% F(Ab')₂ positive cells for CD269-A7D-hu63-IL15/IL15sushi (Figure 11B). These CAR T cells were used in the following in vitro killing assays.

CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cells are able to lyse tumor cell lines expressing either CD269 or CS1 antigens in in vitro assays

CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cells from Figure 11B were assayed for their ability to specifically lyse both K562 cells synthetically expressing either CD269 (BCMA) or CS1 (CD319) antigen. Wild-type K562 cells were transduced with lentiviral vector for CD269 antigen or CS1 antigen expression, and positively selected by FACS (FACS-Aria, BD). Co-

cultures with either K562-BCMAxp or K562-CS1xp synthetic expression cells were set up at 2:1 and 5:1 effector cell:target cell ratios, for 48 hours. Following this incubation, cells were stained using mouse anti-human CD3 antibody (in all cases), and either mouse anti-human CD269 or CS1, and analyzed by flow cytometry. At the 2:1 E:T ratio, 58% of the K562-BCMAxp tumor cells were lysed, while at the 5:1 ratio, 91% tumor cells were lysed (Figure 11C). For co-cultures with K562-CS1-xp tumor cells, the percent lysis was 33 and 72%, respectively (Figure 11C). These results demonstrate that each CAR component of the CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cell is able to lyse its intended target cells.

CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cells exhibit significant anti-tumor activity in xenogeneic mouse model

In order to evaluate the specific *in vivo* anti-tumor activity of CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cells against human tumor cell lines, we developed a xenogeneic mouse model using NSG mice sublethally irradiated and intravenously injected with 4×10^6 of luciferase-expressing MM.1S wild type multiple myeloma cells, to induce measurable tumor formation. Eight days following tumor cell injection, all mice were intravenously injected with a course of 15×10^6 of either control T cells or CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cells. On Day 8 (the day before T cell treatment), and Day 12 (72 hours after treatment), mice were subjected to IVIS imaging to measure tumor burden. Average light intensity measured for the MM.1S mice injected with CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cells was compared to that of mice injected with the control T cells to determine percent lysis of targeted cells. Results showed that only 3 days following treatment with T cells (Day 12), mice treated with CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cells had 90% lower tumor burden than mice given control T cells (Figure 11D). These results show the efficacy of CD269-A7D-CS1-

hu63-IL15/IL15sushi CAR T cells against multiple myeloma cell line in vivo. In addition, blood drawn at sacrifice showed that MM.1S mice injected with CD269-A7D-CS1-hu63-IL15/IL15sushi CAR T cells had a significant higher level of human IL-15/IL-15sushi than the control mice which were undetectable.

5 **Function of IL15 in CD269-A7D-CS1-hu63-IL15/IL15sushi CAR NK cells.**

To further determine if IL-15/IL15sushi is being secreted, NK-92 cell line was transduced with lentiviral vector containing CD269-A7D-CS1-hu63-IL15/IL15sushi CAR. Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype (Figure 11E). Sorted cells were expanded, and after 72 hours supernatant was collected and subjected to
10 ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained on a plate reader were compared to a standard curve generated with human IL-15 to determine concentration of IL-15 in the supernatant. It was determined that IL-15 was detected in the supernatant at 285.9 pg/mL (Figure 11F). By comparison, supernatant containing the same number of wild-type control NK-92 cells had a
15 concentration of only 0.33 pg/mL.

IL15/IL15sushi secreted from CD269-A7D-CS1-hu63-IL15/IL15sushi CAR NK cells can substitute for the function of IL-2 in vitro for T cell expansion

NK-92 cell culture requires the presence of IL-2. IL-15 can replace the absence of IL-2 for NK-92 cell growth or expansion in vitro. This system was used to test the function of IL-
20 15/IL-15sushi fusion secreting from CD269-A7D-CS1-hu63-IL15/IL15sushi CAR transduced NK cells. Sorted CD269-A7D-CS1-hu63-IL15/IL15sushi CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5 x 10⁶ cells per mL, in 1 mL total volume. Cells were added to duplicate wells; one well of each pair contained IL-2 at 300 IU/mL, the other well

did not. After 48 hours (Day 2), cells were counted, and the volume increased to yield a concentration of approximately 0.5×10^6 cells/mL. This process was repeated on Days 4, 6, and 8. As shown in the graph in figure 11E, CD269-A7D-CS1-hu63-IL15/IL15sushi NK CAR cells cultured for 8 days *without* IL-2 in the culture expanded at the same rate as wild-type NK-92 cells cultured *with* IL-2 added, whereas wild-type NK-92 cultured *without* IL-2 had all died by Day 6. This indicates that IL-15 secreted by the NK CAR cells can substitute for the expansion activity of IL-2.

In one embodiment, the engineered cell includes a BCMA-CS1 cCAR polypeptide and IL-15/IL-15sushi (SEQ ID NO. 42), and corresponding nucleotides (SEQ ID NO. 43).

Examples for targeting CD123+ and/or CD33+ leukemia/lymphomas by CD123b-CD33b cCAR (a version of CD123-CD33 cCAR) T cells

Generation of CD123b-CD33b cCAR T-cells

Lentivirus transfected cytotoxic effector T-cells were engineered to express two complete units of CAR linked by a self-cleaving P2A peptide (Figure 12A). The resulting compound CAR (CD123b-CD33b cCAR) is capable of targeting CD123+ and /or CD33+ leukemic cells (Figure 12B). A leader, a scFv, a hinge domain (H), a transmembrane domain (TM), a co-stimulatory domain (CD28 or 4-1BB) and the intracellular signaling domain CD3 zeta (CD3) are included in each CAR unit. A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the CD123b-CD33b cCAR molecule on the T-cell surface.

CD123b-CD33b cCAR T-cell Transduction Efficiency

To evaluate CD123b-CD33b cCAR expression levels on the T-cell surface after transduction, flow cytometry analysis was used (Figure 13). The transduction efficiency was determined to be 25%.

CD123b-CD33b cCAR T-cells effectively lyse acute myeloid leukemia cell lines

To evaluate the anti-tumor activity of the CD123b-CD33b cCAR (CD123b-CD33b cCAR) T-cells, we performed co-cultures using the AML cell line MOLM13 (CD33+CD123+) and the promonocytic U937 cell line (CD33+CD123-). To distinguish between the target leukemia cells (MOLM13 and U937; both are CD3-) and effector T-cells (CD3+) during flow cytometry, cells were stained with CD3. Co-culture assays were performed at effector to target (E:T) ratios of 2:1 and 5:1 for 24 hours, and flow cytometry analysis was used to determine cell lysis rates by CD123b-CD33b cCAR T-cells or control T-cells (Figure 14A, 14B). At the 2:1 E:T ratio, CD123b-CD33b cCAR T-cells were able to lyse around 98% of CD123+CD33+ MOLM13 cells and 99.9% of CD33+ U937 cells when compared to control T-cells. Furthermore, at the 5:1 ratio, 100% lysis of both cell lines was observed (Figure 14C). We also validated the surface markers expressed on both the MOLM13 and U937 cell lines (Figure 14C). Overall, these results suggest that CD123b-CD33b cCAR T-cells specifically and robustly eliminate tumor cells expressing either or both antigens. Moreover, the finding that the CD123b-CD33b cCAR T-cells effectively ablated U937 cells expressing only CD33 and not CD123 supports the fact that each discrete unit of the compound CAR can independently target its antigen and eliminate a target expressing only one antigen or both antigens.

We further evaluated the dose-dependent tumor lysis ability of the CD123b-CD33b cCAR T-cells by varying and decreasing the E:T ratio against two other cell lines: KG1a (CD123dimCD33+) and HL60 (CD123dimCD33+). CD123b-CD33b cCAR T-cells were cultured against KG1a and HL60 cell lines in 0.25:1, 0.5:1, 1:1, 2:1, 5:1, and 10:1 E:T ratios, showing over 75% tumor lysis ability at even a 0.25:1 ratio. Overall, there was a strong correlation between dose and tumor-lysis until saturation at the 5:1 ratio (Figure 14D).

CD123b-CD33b cCAR T-cells effectively lyse primary myeloid leukemia tumor cells

We next established the anti-tumor properties of the CD123b-CD33b cCAR T-cells against primary tumor cells. Cells were stained with CD3 to distinguish the CAR T-cells from the CD3- leukemia samples. Different primary patient leukemia samples including two CD123+CD33+ AML and two CD123+ B-ALL samples (PT1:AML, PT2:B-ALL, PT3:AML, and PT4:B-ALL) were assayed in this panel and flow cytometry analysis was performed to verify tumor lysis with depleted target populations encircled (Figure 15). Compared to the previous anti-tumor cytotoxicity results for AML cell lines (Figure 14), CD123b-CD33b cCAR

T-cells showed similarly positive results against all patient samples, with over 80% tumor lysis at the 2:1 ratio and more than 98% tumor lysis at the 5:1 E:T ratio (Figure 15). Moreover, similarly to our cell lines, the finding that the CD123b-CD33b cCAR T-cells effectively ablated PT2 cells expressing only CD123 and not CD33 supports the fact that each discrete unit of the compound CAR can independently target its antigen and eliminate a cell expressing only one of its target antigens (as seen against CD33+ U937 and CD123+ PT2 cells) or both target antigens (as seen against CD123+CD33+ MOLM13 and PT1 cells). Overall, these results suggest that CD123b-CD33b cCAR T-cells display high killing efficacy against patient tumor cells expressing either or both antigens.

We also specifically examined the ability of our CD123b-CD33b cCAR to eliminate specific cell populations including leukemic stem cells (CD123+CD34+CD38-) in the PT3 sample and myeloid leukemia bulk disease (CD34variableCD33+) in the PT4 sample (Figure 15C, 15D). We found that CD123b-CD33b cCAR T-cells successfully ablated both LSCs and bulk disease cells.

CD123b-CD33b cCAR T-cells' discrete receptor units independently lyse target cells in an antigen-specific manner

To further confirm our cCAR's independent antigen targeting ability, we generated Jurkat artificial cell lines expressing either CD123 or CD33 and tested CD123b-CD33b cCAR T-cells against these cells in addition to wild-type Jurkat cells expressing neither antigen (Figure 16). We found that the CD123b-CD33b cCAR T-cells specifically and potently ablated cells expressing either the CD123 or CD33 antigen when compared to wild-type Jurkat cells expressing neither antigen (Figure 16A, 16B and 16C). Overall, we conclude that the our CD123b-CD33b cCAR T-cells can act via stimulation of either CAR receptor, and are able to target cells expressing only one target antigen or both equally well, and eliminate targets with high efficacy.

CD123b-CD33b cCAR T-cells exhibit profound anti-tumor activity in two xenograft mouse models of AML using MOLM13 and U937 cells

In order to evaluate the in vivo anti-tumor activity of CD123b-CD33b cCAR T-cells as a predictor of their therapeutic efficacy in patients, we developed two xenograft mouse models

(Figure 17). NSG mice were sublethally (2.0 Gy) irradiated and intravenously injected with either 1.0×10^6 firefly luciferase-expressing MOLM13 cells or 1.0×10^6 firefly luciferase-expressing U937 cells. On day 4 following MOLM13 or U937 engraftment, mice were intravenously injected with a 10×10^6 cells of either CD123b-CD33b cCAR or control T-cells.

- 5 To evaluate tumor burden in mice, RediJect D-Luciferin (Perkin-Elmer) was injected intraperitoneally on days 6, 9, and 13, and mice were subjected to IVIS imaging to quantify the luciferase activity (Caliper LifeSciences) (Figure 17A, 17B). As observed by IVIS imaging, total flux levels continually increased in control mice with drastic tumor burden growth. In contrast, CD123b-CD33b cCAR treated mice significantly suppressed tumor burden as early as day 3. By
- 10 day 6, mice treated with the cCAR had over 80% reduction in tumor burden in both models (Figure 17A, 17B). This tumor suppression was maintained and increased in potency through day 13, as total flux in CD123b-CD33b cCAR treated mice remained near background null values with statistically significant differences from control T-cell treated mice.

We also evaluated tumor cell and CAR T-cell persistency at the time of sacrifice.

- 15 Peripheral blood was collected from each experimental mouse at the time of sacrifice along with control mice, and analyzed via flow cytometry for the presence of transplanted tumor (MOLM13 or U937 cells) and T-cells (cCAR or control). MOLM13 and U937 cells are CD3⁻ cells, allowing them to be distinguished from CD3⁺ CAR or control T-cells. Murine peripheral blood cells were gated by side scatter and human CD45 antibody, and then broken down into CD3 vs. CD33.
- 20 While control treated mice showed significant residual tumor populations (~75-87%) in the peripheral blood, CD123b-CD33b cCAR treated mice showed virtual depletion of all tumor comparable to control mice (Figure 17C). In addition, CD123b-CD33b cCAR treated mice showed significant T cell expansion with virtually all human cells in the peripheral blood that were CAR T cells. This confirms the potency and persistency of our cCAR T-cells in
- 25 maintaining long-term responses. Furthermore, CD123b-CD33b cCAR treated mice showed significantly increased survival outcomes as compared to control treated mice (Figure 17A, 17B).

In vivo depletion of infused cCAR T-cells following treatment with CAMPATH

- 30 For clinical treatment using CAR T-cells against acute myeloid leukemias, establishment of safety methods to eliminate CAR T-cells from patients may be necessary after tumor depletion

or in emergency cases due to unexpected side effects caused by CAR therapy. T-cells and B-cells express CD52 on the cell surface and a CD52 specific antibody, CAMPATH (alemtuzumab), can eliminate CD52+ cells from circulation. To assess the effect of CAR elimination by CAMPATH treatment, we conducted in vivo procedures as described (Figure 18A). We intravenously
5 injected 10×10^6 cCAR T-cells into irradiated mice. On the next day, we administered 0.1mg/kg of either CAMPATH or PBS via IP injection to 3 mice of each group. At 6 and 24 hours following CAMPATH treatment, we collected peripheral blood and determined the presence of cCAR T-cells by FACS analysis. cCAR T-cells were gated by side scatter (SSC) and CD3 expression and CD3 and CD45 expression to distinguish them from mouse cells. CAMPATH
10 injection depleted cCAR T-cells in blood at both 6 h and 24 h (Figure 18B, 18C). These findings support the use of CAMPATH as a safety switch to rapidly deplete CAR-T cells from the circulation.

In one embodiment, the engineered cell includes a CD123-CD33 cCAR polypeptide, and IL-15/IL-15sushi (SEQ ID NO. 34), and corresponding nucleotides (SEQ ID NO. 35).

15 In one embodiment, the engineered cell includes a CD123-CLL1 cCAR polypeptide, and IL-15/IL-15sushi (SEQ ID NO. 36), and corresponding nucleotides (SEQ ID NO. 37).

Examples for targeting B-ALL and other leukemias by CD19b-CD123 cCAR (a version of CD19-CD123 cCAR)

Generation of CD19b-CD123 cCAR T cells

Lentivirus transfected cytotoxic effector cells, namely T cells, are engineered to express an anti-CD19 single-chain variable fragment (scFv1, CD19b) region fused to an anti-CD123 fragment (scFv2, CD123) by a self-cleaving P2A peptide. These antibody domains are linked by
25 CD8-derived hinge (H) and transmembrane (TM) regions to 4-1BB and CD28 co-activation domains and a CD3 ζ signaling domain (Figure 19). A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the CD19b-CD123 cCAR molecule on the T-cell surface.

CD19b-CD123 cCAR T-cell Transduction Efficiency

T-cells isolated from umbilical cord blood (UCB) buffy coats were transduced with CD19b-CD123 cCAR lentivirus after 2 days of activation. The CD19b-CD123 cCAR transduction efficiency was determined to be 26% by flow cytometry (Figure 20).

5

CD19b-CD123 cCAR-2G T-cells effectively lyse CD19-positive and CD123-positive leukemic cell lines

To assess the cytotoxicity of CD19b-CD123 cCAR T-cells, we conducted co-culture assays at a 5:1 effector:target (E:T) ratio against leukemia/lymphoma cell lines with artificially
10 expressing CD19 and CD123. K562 cells (a myeloid leukemia cell line) were used to express CD19 antigen by lentiviral infection (named K19), and wild type K562 cell line was used as a control. Jurkat cells were similarly used to express CD123 antigen (named J123), and wild-type Jurkat cells were used as a control. CD19b-CD123 cCAR T-cells lysis of target cells was quantified by flow cytometry. In 16 hour co-cultures, CD19b-CD123 cCAR T-cells lysed over
15 66% of K19 cells at 16 hours, and over 99% at 48 hours (Figure 21A). Over 88% of J123 cells were lysed at 16 hours, reaching saturation (Figure 21B and 21D). Control K562 and control Jurkat cells were not significantly lysed, with less than 20% lysis. The finding that the CD19b-CD123 cCAR T-cells effectively ablate both artificially-induced singly-positive CD19 and CD123 cells supports the idea that each discrete unit of the compound CAR can independently
20 target its antigen and eliminate a target expressing only one antigen or both antigens. Furthermore, the lack of cell lysis of control K562 and Jurkat cells demonstrates that CD19b-CD123 cCAR T-cells exhibit antigen-specific cytotoxicity.

We next assessed the ability of CD19b-CD123 cCAR T-cells to target leukemia/lymphoma cell lines with naturally occurring CD19/CD123 antigen expression: human
25 mantle cell lymphoma SP53 (CD19⁺ CD123⁻) and human acute myeloid leukemia KG1a (CD19⁻ CD123⁺). In 16 hour co-cultures, the CD19b-CD123 cCAR exhibited virtually complete lysis of SP53 cells, with 86% depletion of target cells, reaching saturation (Fig. 21C). In KG1a, CD19b-CD123 cCAR lysed over 69% of CD123⁺ target cells at 16 hours, and over 94% at 48 hours (Fig. 21C and 21D). Overall, CD19b-CD123 cCAR T-cells specifically
30 and effectively lysed target populations expressing either antigen target, displaying effective bulk cytotoxicity.

CD19b-CD123 cCAR-2G T-cells effectively lyse primary B-cell Acute Lymphoblastic Leukemia (B-ALL) and Acute Myeloid Leukemia (AML) tumor cells

We conducted co-cultures using CD19b-CD123 cCAR T-cells against primary tumor cells to evaluate their ability to kill diverse primary leukemia cell types. Patient samples were stained with CMTMR Cytotracker Dye to distinguish primary tumor cells from CAR T-cells. Co-cultures were performed with two samples, PT1:B-ALL and PT2:AML, and flow cytometry was performed to verify tumor-lysis. Flow cytometry analysis of the PT1 sample showed a near complete CD19+ phenotype, with a distinct CD19+ CD123+ population. The PT2 sample showed a mixed tumor phenotype with a partial CD123+ CD19- phenotype (Figure 22A). CD19b-CD123 cCAR T-cells showed robust ablation of the PT1 primary B-ALL sample, with near complete lysis at an E:T ratio of 5:1 at 24 hours (Figure 22B and 22D). CD19b-CD123 cCAR T-cells also ablated the PT2 primary AML sample, with 31% lysis at 24 hours and 67% lysis at 48 hours (Figure 22C and 22D). In summary, CD19b-CD123 cCAR T cells exhibited robust anti-tumor activity against both leukemia cell lines and primary tumor cells expressing different combinations of CD19 and CD123 (Figure 22D).

CD19b-CD123 cCAR-3G T-cells exhibit profound anti-tumor activity in two xenograft mouse models of AML and B-ALL using MOLM-13 and REH cells.

In order to evaluate the in vivo anti-tumor activity of CD19b-CD123 cCAR T-cells, we developed two models, one with luciferase-expressing MOLM13 cells (CD123+ CD19-), and one with luciferase-expressing REH cells (CD19+ CD123-) to induce measurable tumor formation. Mice were given a single dose of CD19b-CD123 cCAR T-cells or control GFP cells, and tumor burden was measured on days 3, 6, 8 and 11 (Fig. 23A). In the MOLM13 model, there was a significant difference ($P < 0.01$) between the cCAR treated and control groups by day 6, with less light intensity and thus less tumor burden in the CD19b-CD123 cCAR T-cell injected group compared to control (Fig. 23B). Mice injected with CD19b-CD123 CAR T-cells had 99% less tumor burden than control mice by day 11. Next, we compared mouse survival across the two groups. Following the IVIS imaging experiments previously described, mice were observed every day for symptoms of severe illness and were sacrificed once movement was greatly

impaired. All control mice died by day 18, while the CD19b-CD123 CAR T treated mice survived longer than control mice by up to 15 days ($p=0.0031$) (Figure 23C).

0A1 similar result was seen in the REH mouse model (Figure 23D). REH leukemic mice injected with CD19b-CD123 cCAR T cells had 99% less tumor burden than control mice on day 16 (Figure 23E). When comparing mouse survival across cCAR and control treated groups, CD19b-CD123 cCAR T injected mice survived much longer than control mice (Figure 23F) ($p=0.0031$). In summary, these in vivo data indicate that CD19b-CD123 cCAR T-cells significantly reduce tumor burden and prolong survival in MOLM13-injected and REH-injected NSG mice when compared to control T-cells.

Screening and Evaluation of Several versions of cCARs targeting BCMA+ and /or CS1+ leukemic cells, particularly multiple myeloma cells using co-culture killing assays.

1. Generations of different versions of BCMA (CD269)-CS1 cCARs.

As described above, creation of compound CARs bearing different CAR units can be quite challenging. We selected various CAR body elements to express multiple units of CARs in a single vector using a strong promoter and P2A self-cleaving site. The hinge region in the CAR was chosen so that interaction of the hinge region between each CAR unit could be avoided. Lentivirus transfected cytotoxic effector cells, namely T cells, were engineered to express an anti-BCMA (CD269) single-chain variable fragment (scFv1) region fused to an anti-CS1 fragment (scFv2) by a self-cleaving P2A peptide. These scFv domains are linked by CD8-derived hinge (H) and transmembrane (TM) regions to 4-1BB and CD28 co-activation domains and a CD3 ζ (CD3) signaling domain (Figure 30). A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the compound CAR molecule on the T-cell surface. Finally, the generated constructs were screened and evaluated for their expression and functions. scFv1 represents different scFv versions (A7D or C11D) against BCMA antigen. scFv 2 represents different scFv versions (hu63 or mu34 or mu90) against CS1 antigen.

2. Varied level of CAR expression in T cells transduced with various versions of BCMA-CS1 cCAR lentiviruses.

Peripheral blood mononuclear buffy coat cells were activated for three days and transduced with the lentiviral vector for 6 different sequence variations cCARs comprised of CD269 (A7D or C11D) combined with CS1 (hu63, mu34 or mu90) CAR, or control vector. Expression of CAR on the T-cell surface was demonstrated three days after transduction by staining transduced T cells with goat anti-mouse Fab antibody and mouse anti-human CD3. Figure 30A shows surface expression for each of the CD269-CS1 CARs: for A7D-mu34, 11.2%; A7D-mu90, 23.1%; A7D-hu63, 28.5%; C11D-mu34, 28.0%; C11Dmu90 13.6%; and C11Dhu63, 42%. This demonstrates the need to find a pairing of CAR units that result in the highest level of CAR expression. A high efficiency lentiviral packaging cell line is critical for generation of a high titer for these constructs (Figure 30B). We used lenti-X 293 T cell line as a packaging system to generate high viral titers for compound CAR constructs. Lenti-X 293T packaging cell line clearly outperformed the other cell lines and produced over 2 to 6-times as many viruses as 293 FT cells.

The transduction efficiency (percentage of CAR T cells) for cCARs is often lower than for a single-unit CAR. There are several ways to improve efficiency, at both the transfection and transduction steps. To improve viral titer for making cCARs, it is preferred to use LentiX™ 293 T (Clontech/Takara) packaging cell line, which is selected for high titer lentivirus production, instead of the commonly used HEK-293FT. It is also preferable to increase the amount of plasmid DNA (containing the cCAR construct) 1.5- to 2.0-fold when transfecting packaging cells, to increase transfection efficiency. The amount of viral packaging plasmids and transfection reagent remains the same during the forming of complexes. Transduction efficiency can be further enhanced by lowering the ratio of T cells to viral vector during the transduction step, to 0.3×10^6 cells per mL, and increasing the volume of lentiviral supernatant or lentiviruses.

3. Testing CAR expression in T cells transduced with various anti-BCMA lentiviral vectors.

Based on the above studies, CD269-A7D (also called A7D) and CS1-hu63 (also called hu63) were chose as good candidates for generation of enhanced CARs or compound CAR (cCAR). We also generated a cCAR (CD269-A7D-C11D-2G) targeting two epitopes on the same antigen, BCMA. In this cCAR, each unit of CARs bears different scFv targeting different epitopes of BCMA. Enhanced CARs are CD269-A7D-IL15/IL15sushi and CD269-A7D-41BBL-2G targeting BCMA antigen. Compound CARs are CD269-A7D-CD19b-2G targeting

BCMA and CD19 antigens, and CD269-A7D-CS1-hu63 or CD269-C11D-CS1-hu63-BB targeting BCMA and CS1 antigens.

Peripheral blood mononuclear buffy coat cells were activated for three days and transduced with the anti-BCMA lentiviral vectors for single CARs (CD269-A7D-2G, CD269-A7D-IL15/IL15sushi, CD269-A7D-41BBL-2G) and cCARs (CD269-A7D-C11D-2G, CD269-A7D-CD19b-2G, CD269-A7D-CS1-hu63, CD269-C11D-CS1-hu63-BB) or control vector (Figure 30B). Expression of CAR on the T-cell surface was demonstrated three days after transduction by staining transduced T cells with goat anti-mouse Fab antibody and mouse anti-human CD3. Figure 30B shows surface expression for each of the lentiviral CARs: for CD269-A7D-2G, 48.4%; CD269-A7D-IL15/IL15sushi, 32.2%; CD269-A7D-41BBL-2G, 36%; CD269-A7D-C11D-2G, 27.4%; CD269-A7D-CD19b-2G, 30.6%; CD269-A7D-CS1-hu63, 28.5%; and CD269-C11D-CS1-hu63-BB, 42.0%.

4. CD269-A7D-CD19b cCAR T cells efficiently lyse both BCMA and/or CD19-expressing tumor cell lines

The CD269-A7D-CD19b cCAR T cells were tested for their ability to lyse individual target cell lines in in vitro co-culture assays (Figure 30C and 30D). K562 cells were modified to synthetically express either BCMA (CD269) (called K-BCMA) or CD19 (called K-19) on the cell surface. After 18-hour co-incubation, cells were labeled with anti-human CD3 and either anti-human CD269 or CD19, and analyzed by flow cytometry (Figure 30C and CD30E). CD269-A7D-CD19b cCAR T cells were able to lyse 31% of the target K-BCMA cells at the 2:1 E:T ratio, and 65% at 5:1 ratio. CD269-A7D-CD19b cCAR T cells were also able to lyse 60% of the target K-CD19 cells at the 2:1 E:T ratio, and nearly all at 5:1 ratio (Figure 30D and CD30E). These results confirm that each CAR unit – CD269 and CD19b CAR – effectively lyses its specific target cells.

5. CD269-A7D-41BBL, CD269-A7D-CS1-hu63, and CD269-A7D-C11D cCAR T cells efficiently lyse MM1S tumor cell line

Various versions of BCMA-CS1 cCAR T cells generated above were tested for their ability to lyse specific target cell lines in in vitro co-culture assays. The human multiple myeloma cell line, MM1S, was co-cultured with CD269-A7D-41BBL CAR, CD269-A7D-CS1-

hu63 cCAR, CD269-A7D-C11D cCAR T cells, or control T cells, at 2:1 and 5:1 E:T ratios (Figure 30F). After 18-hour co-incubation, cells were labeled with CMTMR (Cell Tracker) and anti-human CD269 and analyzed by flow cytometry. CD269-A7D-41BBL CAR T cells were able to lyse 74% of the target MM1S cells at the 2:1 E:T ratio, and 90% at 5:1 ratio, while
5 CD269-A7D-CS1-hu63 cCAR T cells lysed 59% and 90%, and CD269-A7D-C11D CAR T cells lysed 62% and 86% of the MM1S cells at 2:1 and 5:1 ratios, respectively (Figure 30F). These compound CARs did not appear to show any evidence of the CAR to CAR interaction. In vivo anti-tumor activities, cell killing is performed in a xenogeneic mouse model and targeted cells expressing BCMA or CS1 or both are eliminated or suppressed by cCAR T or NK cells
10 using methods described in PCT/US2016/019953 and PCT/US2016/039306

6. CD269-A7D-41BBL, CD269-A7D-CS1-hu63, and CD269-A7D-C11D CAR T cells efficiently lyse the cell line K562 synthetically expressing BCMA or CS1

Various versions of BCMA-CS1 cCAR T cells generated above were tested for their
15 ability to lyse specific target cell lines in in vitro co-culture assays. K562 cells were modified to synthetically express either BCMA (CD269) or CS1 on the cell surface, and were subsequently co-cultured with CD269-A7D-41BBL, CD269-A7D-CS1-hu63, CD269-A7D-C11D cCAR T cells, or control T cells, at 2:1 and 5:1 E:T ratios. After 18 hour co-incubation, cells were labeled with anti-human CD3 and anti-human CD269 (or CS1) and analyzed by flow cytometry. CD269-
20 A7D-41BBL CAR T cells were able to lyse 56% of the target K-BCMA cells at the 2:1 E:T ratio, and completely eliminated all target cells at 5:1 ratio, while CD269-A7D-CS1-hu63 cCAR T cells lysed 38% and 79%, and CD269-A7D-C11D CAR T cells lysed 16% and 74% of the K-BCMA cells at 2:1 and 5:1 ratios, respectively (Fig. 30G). Only CD269-A7D-CS1-hu63, CD269-A7D-C11D cCAR T cells were tested in co-culture against the K-CS1 cells (Figure 30H).
25 CD269-A7D-CS1-hu63 cCAR T cells lysed 18% and 54%, of the K-562 cells at 2:1 and 5:1 ratios, respectively, while the CD269-A7D-C11D cCAR T cells, a compound CARs targeting two different epitopes on the BCMA antigen, showed no ability to lyse the K-CS1 cells at either ratio, which was expected, due to the absence of a CS1 CAR unit. (Figure 30H). These results demonstrate the ability of each CAR unit to specifically lyse its target population.

Examples for targeting CLL1+ and/or CD33+ leukemic cells by CLL1-CD33b cCAR (a version of CLL1-CD33)

Transduced T cells efficiently express the CLL1-CD33b cCAR (CLL1-CD33b CAR)

Peripheral blood mononuclear buffy coat cells were activated for two or three days and
5 transduced with either CLL1-CD33b cCAR or control vector. Expression of CLL1-CD33b
cCAR on the T-cell surface was demonstrated three days after transduction by staining
transduced T cells with goat anti-mouse Fab antibody and mouse anti-human CD3. Figure 31
shows that 29.7% of cells transduced with the CLL1-CD33b cCAR viruses were positive for
both F(ab')₂ and CD3 as determined by flow cytometry.

CLL1-CD33b cCAR T cells specifically target both CLL1 (CLL-1) and CD33-expressing tumor cell lines

T cell coculture killing assays were performed to determine the ability of CLL1-CD33b
cCAR T cells to effectively and specifically lyse CLL1 (CLL-1) and CD33-expressing cell lines:
15 the acute myeloid leukemia cell line HL60, which expresses both antigens on the cell surface
naturally; and Jurkat cells which were modified to synthetically express either CLL1 (called
Jurkat-CLL-1xp) or CD33 (called Jurkat-CD33xp). In addition, CLL1-CD33b cCAR T cells
were co-cultured against the REH and CCRF-CEM cell lines, which are negative for CLL1 and
CD33 (Figure 32A and 32B). All target cells were pre-labeled with CFSE membrane dye to
20 distinguish them from T cells. After 18 hour co-incubation, cells were labeled with anti-human
CD3 and analyzed by flow cytometry. At the low 2:1 effector:target ratio, CLL1-CD33b cCAR T
cells were able to effectively lyse HL60 cells (89%), Jurkat-CLL-1xp cells (84%) and Jurkat –
CD33xp cells (96%) (Figure 32C, 32D and 32E) ; at the 5:1 E:T ratio, nearly all target cells were
depleted (Fig. 2a-d). However, the REH (8%) and CCRF-CEM cells (14%), both off-target,
25 showed very little cell lysis (Figure 32A and 32B). This demonstrates remarkable potency and
specificity of the CLL1-CD33b cCAR T lysis. The results are summarized in the bar graph (Fig.
32F).

CLL1-CD33b compound CAR T cells are able to demonstrate potent and directed cytotoxicity *in vitro*.

30 We conducted co-culture assays using target AML cell lines HL60 and U937 expressing
high amounts of both CLL-1 and CD33. We found that the CLL-1 CAR T cell was able to
potently ablate both of these cell types at high efficiency >90% (Figure 32G and 32H).

Furthermore, the compound CAR exhibited minimal targeting of negative control cell line CCRF-CEM with basal levels of activity (Figure 32I).

In addition, the CLL1-CD33b cCAR demonstrated potent dose dependent cytotoxicity in an escalating dosage scheme, with ~50% activity even at the lowest dose threshold of 0.25:1 (effector:target) cell ratio (Figure 32J).

Compared to single CAR T options, the CLL1-CD33b cCAR T cells demonstrate superior anti-tumor activity

Jurkat cells expressing either CLL-1 or CD33 were combined in a 1:1 ratio and incubated with 100,000 effector cells for a final effective E:T ratio of 1:2. The results show that the compound CAR exhibited highly specific and potent cytotoxicity against either CLL-1 or CD33 expressing sets of Jurkat cells (>85%) while demonstrating increased cytotoxicity over single CAR options for their respective antigens (Figure 32K and 32L).

CD19b-IL-21 CAR (a version of CD19-IL-21 CAR)

EXAMPLE

An engineered CD19b-IL-21 (CD19b-IL21) CAR cell was prepared in accordance with the present disclosure (Figure 33A). CD19b CAR is equipped with secreting IL-2 to lyse leukemia/lymphoma expressing CD19 antigen.

Peripheral blood mononuclear buffy coat cells were activated for two or three days and transduced with either CD19b-IL-21 or control vector. Expression of CD19b-IL-21 on the T-cell surface was demonstrated three days after transduction by staining transduced T cells with goat anti-mouse Fab antibody and mouse anti-human CD3. Figure 33B shows that 63.9% of cells transduced with the CD19b-IL-21 CAR viruses were positive for both F(ab')₂ and CD3 as determined by flow cytometry.

Cell killing assay is performed and targeted cells expressing CD19 are lysed by IL-19-IL-21 CAR.

In vivo anti-tumor activities, cell killing is performed in a xenogeneic mouse model and targeted cells expressing CD19 are eliminated or suppressed by CD19b-IL-21 CAR T or NK cells using methods described in PCT/US2016/019953 and PCT/US2016/039306

Similar assays can be used for BCMA-IL-18 CAR (Figure 35)

In one embodiment, the engineered cell includes a CD19 chimeric antigen receptor polypeptide and IL-21 (SEQ ID NO. 16), and corresponding nucleotides (SEQ ID NO. 17).

In one embodiment, the engineered cell includes a CD19 chimeric antigen receptor polypeptide and IL-21 anchor (SEQ ID NO. 1), and corresponding nucleotides (SEQ ID NO. 2).

In one embodiment, the engineered cell includes a BCMA chimeric antigen receptor polypeptide and IL-18 (SEQ ID NO. 11), and corresponding nucleotides (SEQ ID NO. 12).

5 In one embodiment, the engineered cell includes a BCMA chimeric antigen receptor polypeptide and IL-18 anchor (SEQ ID NO. 13), and corresponding nucleotides (SEQ ID NO. 14).

CD19b-IL-21 anchor CAR (a version of CD19-IL-21 anchor)

10 **EXAMPLE**

An engineered CD19b-IL-21 anchor (CD19b-IL21) CAR cell was prepared in accordance with the present disclosure (Figure 34). CD19b- IL-21 anchor CAR is to lyse leukemia/lymphoma expressing CD19 antigen.

15 Cell killing assay is performed and targeted cells expressing CD19 are lysed by IL-19-IL-21 anchor CAR.

In vivo anti-tumor activities, cell killing is performed in a xenogeneic mouse model and targeted cells expressing CD19 are eliminated or suppressed by CD19b-IL-21 anchor CAR T or NK cells using methods described in PCT/US2016/019953 and PCT/US2016/039306

Similar assays can be used for BCMA-IL-18 anchor CAR (Figure 36)

Examples for targeting multiple myeloma by BCMA-CD38 cCAR

EXAMPLE

An engineered BCMA-CD38 cCAR cell was prepared in accordance with the present disclosure (Figure 37). Lentivirus transfected cytotoxic effector T or NK -cells were engineered to express two complete units of CAR linked by a self-cleaving P2A peptide. The resulting compound CAR) is capable of targeting BCMA+ and /or CD38+ multiple myeloma cells or abnormal plasma cells (Figure 37). A leader, a scFv, a hinge domain (H), a transmembrane domain (TM), a co-stimulatory domain (CD28 or 4-1BB) and the intracellular signaling domain CD3 zeta (CD3) are included in each CAR unit. A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the BCMA-CD38 cCAR molecule on the T or NK-cell surface.

BCMA-CD38 cCAR is to lyse multiple myeloma cells or abnormal plasma cells expressing BCMA and/or CD38 antigen.

Cell killing assay is performed and targeted cells expressing BCMA and/or CD38 antigen are lysed by BCMA-CD38 cCAR.

In vivo anti-tumor activities, cell killing is performed in a xenogeneic mouse model and targeted cells expressing BCMA and/or CD38 antigen are eliminated or suppressed by BCMA-CD38 cCAR T or NK cells using methods described in PCT/US2016/019953 and PCT/US2016/039306.

In one embodiment, the CD38 antigen recognition domain includes SEQ ID NO. 15.

In one embodiment, the engineered cell includes a first chimeric antigen receptor polypeptide having a BCMA antigen recognition domain and second chimeric antigen receptor polypeptide having a CD38 recognition domain. In one embodiment, this engineered cell includes a polypeptide of SEQ ID NO. 5, 7, 9 and corresponding polynucleotide of SEQ ID NO. 6, 8, 10.

Structural organization of CLL1-CD33b-IL-15/IL-15sushi (CLL1-CD33-IL-15/IL-15sushi)

CLL1-CD33b-IL-15/IL-15sushi (Figure 39A) contains two independent units of CARs, CLL-1 CAR(also called anti-CD371 CAR or anti-CLL1 CAR), and CD33b CAR (also called anti-CD33 CAR). CLL1-CD33b-IL-15/IL-15sushi is able to secrete IL-15/IL-15sushi. The soluble

IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK or NK T cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion can enhance CAR T/NK or NK T cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

CAR Expression

Activated human peripheral blood T cells were transduced with the lentiviral vector from CLL1-CD33b-IL15/IL15sushi. CAR. Figure 39B shows the transduction efficiency between activated T cells transduced with either control vector, or CLL1-CD33b-IL15/IL-15sushi CAR vector, as determined by labeling with goat anti-mouse F(Ab')₂ antibody. Activated T cells transduced with the CAR vectors resulted in 22% F(Ab')₂ positive cells for CLL1-CD33b-IL15/IL15sushi (Figure 39B). These CAR T cells were used in the following in vitro killing assays.

CLL1-CD33b-IL15/IL15sushi CAR T cells are able to lyse tumor cell lines expressing either CLL-1 or CD33 antigens in in vitro assays

CLL1-CD33b-IL15/IL15sushi CAR T cells from Figure 39B were assayed for their ability to specifically lyse REH cells synthetically expressing either CLL-1 antigen or CD33 antigen. Wild-type REH cells were transduced with a lentiviral vector for CLL-1 or CD33 antigen expression and positively selected by FACS (FACS-Aria, BD) to create REH-CLL1xp and REH-CD33xp cell lines. Co-cultures with control T cells or CLL1-CD33b-IL15/IL-15sushi CAR T cells, and either REH-CLL1xp (REH cells expressing CLL-1 antigen) or REH-CD33xp (REH cells expressing CD33 antigen) cells were set up at 2:1 and 5:1 effector cell:target cell ratios, for 24 hours. Following this incubation, cells were stained using mouse anti-human CD3 antibody (in all cases), and either mouse anti-human CLL1 or CD33, and analyzed by flow cytometry. At the 2:1 E:T ratio, 76% of the REH-CLL1xp tumor cells were lysed, while at the 5:1 ratio, 94% tumor cells were lysed (Figure 39C). For co-cultures with REH-CD33xp cells, the lysis was also robust, 66.7% for 2:1 and 96% for 5:1 (Figure 39C). These results demonstrate that each CAR component of the CLL1-CD33b-IL15/IL15sushi CAR T cell is able to lyse its intended target cells.

CLL1-CD33b-IL15/IL15sushi CAR T cells exhibit significant anti-tumor activity in xenogeneic mouse model

In order to evaluate the specific *in vivo* anti-tumor activity of CLL1-CD33b-IL15/IL-15sushi CAR T cells against human tumor cell lines, we developed a xenogeneic mouse model using NSG mice sublethally irradiated and intravenously injected with 1×10^6 of luciferase-expressing MOLM13 wild type acute myeloid leukemia tumor cells, which express CD33 on the cell surface, to induce measurable tumor formation. Four days following tumor cell injection, all mice were intravenously injected with a course of 15×10^6 of either control T cells or CLL1-CD33b-IL15/IL-15sushi CAR T cells. On Day 4 (the day before T cell treatment), and days 8 (72 hours after treatment), and 12, mice were subjected to IVIS imaging to measure tumor burden. Average light intensity measured for the MOLM13 mice injected with CLL1-CD33b-IL15/IL15sushi CAR T cells was compared to that of mice injected with the control T cells to determine percent lysis of targeted cells. Results showed that only 3 days following treatment with T cells (Day 8), mice treated with CLL1-CD33b-IL15/IL15sushi CAR T cells had 63% less (dorsal view) and 59% (ventral view) lower tumor burden than mice given control T cells (Figure 40A, 40B). By Day 12, percent lysis had increased to 77% and 86%, respectively. These results show the efficacy of CLL1-CD33b-IL15/IL15sushi CAR T cells against an AML cell line *in vivo*.

IL-15/IL15sushi secretion results in persistence of CLL1-CD33b-IL15/IL15sushi CAR T cells in peripheral blood of mice

Blood was drawn from each of the 4 mice at sacrifice; each of the control mice was euthanized on Day 18, once the animals had exhibited signs of paralysis due to spreading of the MOLM13 tumor cells to various organs. The two CAR T cell treated mice were euthanized on Days 21 and 23. Peripheral blood was collected and labeled with mouse anti-human CD45, CD3 and CD33, and subjected to flow cytometry. Transplanted human cells were gated by CD45, and analyzed for T cell and MOLM13 cell populations. As shown in Figure 40C, control mice had a large population of tumor cells (blue dots), while the blood of CAR T -treated mice appeared to be nearly tumor-free. Despite the lack of tumor cells in the blood, these mice still maintained large populations of CAR T cells (green dots), an indication of CAR T cell persistence, and protection of the mouse from engraftment of MOLM13 tumor cells sequestered in other organs. To determine if this persistence could be due to secretion of IL15/IL-15sushi, the plasma from

each mouse was subjected to ELISA to quantify the amount of secreted human IL-15 fusion. As shown in figure 40C, IL-15 was not detected in the two control mice, but low level concentrations of 30 and 40 pg/mL was detected in CAR T cell treated mice.

Function of IL15 in CLL1-CD33b-IL15/IL15sushi CAR NK cells.

5 To determine if IL-15 is being secreted, NK-92 cell line was transduced with lentiviral vector containing CLL1-CD33b-IL15/IL-15sushi CAR. Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype (Figure 40D). Sorted cells were expanded, and after 72 hours supernatant was collected and subjected to ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained
10 on a plate reader were compared to a standard curve generated with human IL-15 to determine concentration of IL-15 in the supernatant (Figure 40E). It was determined that IL-15 was detected in the supernatant at 488 pg/mL. By comparison, supernatant containing approximately the same number of wild-type control NK-92 cells had a background concentration of only 0.33 pg/mL.

15 IL15/IL-15sushi secreted from CLL1-CD33b-IL15/IL15sushi CAR NK cells can substitute for the function of IL-2 in vitro

Sorted CLL1-CD33b-IL15/IL15sushi CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5 x 10⁶ cells per mL, in 1 mL total volume. Cells were added to duplicate wells; one well of each pair contained IL-2 at 300 IU/mL, the other well did not. After
20 48 hours (Day 2), cells were counted, and the volume increased to yield a concentration of approximately 0.5 x 10⁶ cells/mL. This process was repeated on Days 4, 6 and 8. As shown in the graph in figure 40D, CLL1-CD33b-IL15/IL15sushi NK CAR cells cultured for 8 days *without* IL-2 in the culture expanded at close to the same rate as wild-type NK-92 cells cultured *with* IL-2 added, whereas wild-type NK-92 cultured *without* IL-2 had all died by Day 6. This
25 indicates that IL-15 secreted by the NK CAR cells can substitute for the expansion activity of IL-2.

Generation and characterization of CD20cCD19b and CD20hCD19b CARs

The construct comprises a SFFV promoter driving the expression of multiple modular units of CARs linked by a P2A peptide. Upon cleavage of the linker, the cCARs,
30 CD20h-CD19b cCAR or CD20h-CD19b cCAR split and engage upon targets expressing CD20 and/or CD19. As a novel cCAR construct, the activation domains of the construct may

include, but is not limited to, 4-1BB on the CD20h CAR or CD20c CAR segment, and a CD28 region on the CD19b CAR segment. The CD20h CAR section in the cCAR contains a humanized anti-CD20 scFv targeting CD20 expressing cells.

5 Two versions of compound CARs of CD20-CD19 targeting CD20 and/or CD19 expressing cells are CD20c-CD19b (CD20cCD19b) and CD20h-CD19b CAR (CD20hCD19b) used a similar method described above. The percent expression of two compound CARs, CD20cCD19b and CD20hCD19b CAR on transduced T cells was found to be 22% and 28%, respectively (Figure 41A). Buffy coat cells were activated after 3 days with anti-CD3 antibody.
10 Cells were transduced with either control vector (left), CD20cCD19b or CD20hCD19b CAR (right) lentiviral supernatant. After 3 days of incubation, cells were harvested and labeled for flow cytometry.

To assess the specificity of CD20cCD19b and CD20hCD19b CAR T cells on non-target wild-type K562 cells, co-culture experiments were performed at an effector to target ratio of 2:1
15 or 5:1 for 6 hours and were directly analyzed by flow cytometry for CD3 and CD45 (Fig. 41B). Each assay consisted of K562 target cells alone (right), control T cells (left) and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as blue dots (N=2). CD20cCD19b and CD20hCD19b CAR T cells did not lyse K562 tumor cell line that did not expressing either CD20 or CD19 in co-culture assays.

20 To assess the ability of CD20cCD19b and CD20hCD19b CAR T cells to lyse target cells expressing CD19, co-culture experiments were then performed with target K562 cell line synthetically expressing the CD19 antigen (K-19) at an effector to target ratio of 2:1 or 5:1 for 24 hours and were directly analyzed by flow cytometry for CD19 and CD3 (Figure 41C). Each assay consisted of K562-CD19xp target cells alone (right side), control T cells (left panels)
25 and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as green dots. Both types of compound CAR T cells lysed CD19 synthetically-expressing K562 tumor cell line in co-culture assays.

To assess CD20cCD19b and CD20hCD19b CAR T cells' ability to lyse on-target cells expressing CD20, co-culture experiments were performed with target K562 cell line
30 synthetically expressing the CD20 antigen (K-20xp) at an effector to target ratio of 2:1 or 5:1 for 24 hours and were directly analyzed by flow cytometry for CD20 and CD3 (Figure 41D). Each

assay consisted of K562-CD20xp target cells (K-20xP) alone (right side), control T cells (left panels) and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as purple dots. Both types of compound CAR T cells lysed CD19 or CD20 synthetically-expressing K562 tumor cell line in co-culture assays (Figure 41C and 41D)

5 To assess the specificity of CD20cCD19b and CD20hCD19b CAR T cells on-target REH cells expressing CD19, co-culture experiments were performed with CD19-expressing REH cell lines at an effector to target ratio of 2:1 or 5:1 for 24 hours and were directly analyzed by flow cytometry for CD19 and CD3 (Figure 41E). Each assay consisted of REH target cells alone (right side), control T cells (left panels) and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as orange dots. Both types of compound CAR T
10 cells were found to completely lyse CD19-expressing REH tumor cell line in co-culture assays (Figure 41E).

To assess the ability of CD20cCD19b and CD20hCD19b CAR T cells to lyse on-target cells expressing both CD19 and CD20 antigens, co-culture experiments were also performed
15 with the CD19- and CD20-expressing SP53 B-cell lymphoma cell line at an effector to target ratio of 2:1 or 5:1 for 24 hours and were directly analyzed by flow cytometry for CD19 and CD3 (Figure 41F). Each assay consisted of SP53 target cells alone (right side), control T cells (left panels) and either CD20cCD19b or CD20hCD19b CAR T cells (center panels). Target cells are represented as turquoise dots (N=2). Both types of compound CAR T cells completely lysed
20 SP53 tumor cell line, which expresses both CD19 and CD20 antigens, in co-culture assays.

A summary of the co-culture results is shown in Figure 41G, with K562wt (Wild type) performed at a 6 hour co-culture and the others at 24 hours (N=2). Both compound CAR types exhibited superior on-target lysis relative to the control T cells, with CD20hCD19b-2G CAR T cells demonstrating more robust killing of target K562 cells synthetically expressing the CD20
25 antigen when compared to CD20cCD19b-2G CAR T cells.

To characterize anti-tumor activity of CD20h-CD19 CAR T cells *in vivo*, NSG mice were sublethally irradiated and intravenously injected with 1.0×10^6 luciferase-expressing REH cells (Day 0) to induce measurable tumor formation (Figure 42A, B). Starting 6 days after injection of tumor cells, mice were intravenously injected with a course of 10×10^6 CD20hCD19b CAR T
30 cells or vector control T cells. On days 5, 9 and 12, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. By day 12, CD20h-CD19 CAR T cells

achieved 98% lysis of tumor cells for both dorsal and ventral sides. These results demonstrate that CD20h-CD19 CAR T cells exhibit robust lysis of REH cells expressing the CD19 antigen.

Structural organization of CD20h-CD19b-IL-15/IL-15sushi (CD20hCD19b-IL-15/IL-15sushi)

5 Compound CAR (cCAR), CD20h-CD19b-IL-15/IL-15sushi (Figure 43A) contains two independent units of CARs, CD20h CAR(also called anti-CD20 CAR), and CD19b CAR (also called anti-CD19 CAR). CD20h-CD19b-IL-15/IL-15sushi is able to secret IL-15/IL-15sushi. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. The
10 soluble IL-15/IL-15sushi fusion can enhance CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

CAR expression

15 Activated human peripheral blood T cells were transduced with the lentiviral vector from CD20h-CD19b-IL15/IL15sushi. Figure 43B shows the transduction efficiency between activated T cells transduced with either control vector, or CD20h-CD19b-IL15/IL15sushi CAR vector, as determined by labeling with goat anti-mouse F(Ab')₂ antibody. Activated T cells transduced with the CAR vectors resulted in 25% F(Ab')₂ positive cells for CD20h-CD19b-IL15/IL15sushi
20 (Figure 43B). These CAR T cells were used in the following in vitro killing assays.

CD20h-CD19b-IL15/IL15sushi CAR T cells are able to lyse tumor cell lines expressing either CD20 or CD19 antigens or both in in vitro assays

CD20h-CD19b-IL15/IL15sushi CAR T cells from Figure 43B were assayed for their ability to specifically lyse both U937 cells synthetically expressing CD20(also called U937-
25 CD20xp or U-CD20xp) and REH cells naturally expressing CD19 antigen. Wild-type U937 cells were transduced with lentiviral vector for CD20 antigen expression and positively selected by FACS (U-CD20xp; FACS-Aria, BD). Co-cultures with control T cells or CD20h-CD19b-IL15/IL15sushi CAR T cells, and either U-CD20xp cells, or with REH wild-type cells were set up at 2:1 and 5:1 effector cell:target cell ratios, for 24 hours. Following this incubation, cells
30 were stained using mouse anti-human CD3 antibody (in all cases), and either mouse anti-human CD20 or CD19, and analyzed by flow cytometry. At the 2:1 E:T ratio, 77% of the U-CD20xp

tumor cells were lysed, while at the 5:1 ratio, 74% tumor cells were lysed (Figure 43C). For co-cultures with REH tumor cells, the lysis was near complete, 96% for 2:1 and 99% for 5:1 (Figure 43C). These results demonstrate that each CAR component of the CD20h-CD19b-IL15/IL15sushi CAR T cell is able to lyse its intended target cells.

5 **CD20h-CD19b-IL15/IL-15sushi CAR T cells exhibit significant anti-tumor activity in xenogeneic mouse model**

In order to evaluate the specific *in vivo* anti-tumor activity of CD20h-CD19b-IL15/IL15sushi CAR T cells against human tumor cell lines, we developed a xenogeneic mouse model using NSG mice sublethally irradiated and intravenously injected with 1×10^6 of luciferase-expressing REH wild type B-ALL tumor cells, to induce measurable tumor formation. 10 Four days following tumor cell injection, all mice were intravenously injected with a course of 15×10^6 of either control T cells or CD20h-CD19b-IL15/IL-15sushi CAR T cells. On Day 4 (the day before T cell treatment), and days 8 (72 hours after treatment), 12 and 16, mice were subjected to IVIS imaging to measure tumor burden. Average light intensity measured for the 15 REH mice injected with CD20h-CD19b-IL15/IL-15sushi CAR T cells was compared to that of mice injected with the control T cells to determine percent lysis of targeted cells. Results showed that only 3 days following treatment with T cells (Day 8), mice treated with CD20h-CD19b-IL15/IL15sushi CAR T cells had >90% less (dorsal view) and >86% (ventral view) lower tumor burden than mice given control T cells (Figure 44A, B). By Day 16, no tumor could be detected. 20 These results show the efficacy of CD20h-CD19b-IL15/IL15sushi CAR T cells against acute lymphoblastic lymphoma cell line *in vivo*.

IL15/IL-15sushi secretion results in persistence of CD20h-CD19b-IL15/IL-15sushi CAR T cells in peripheral blood of mice

Blood was drawn from each of the 4 mice at sacrifice; each of the control mice was 25 euthanized on Days 28 and 30, once the animals had exhibited signs of paralysis due to spreading of the REH tumor cells to various organs. The two CAR T cell treated mice were euthanized on Days 33 and 36; however, the mouse euthanized on Day 36 was still relatively mobile. Peripheral blood was collected and labeled with mouse anti-human CD45, CD3 and CD19, and subjected to flow cytometry. Transplanted human cells were gated by CD45, and 30 analyzed for T cell and REH cell populations. As shown in Figure 44C, control mice had a large population of tumor cells (blue dots), while the blood of CAR T treated mice appeared to be

tumor-free. Despite the lack of tumor cells in the blood, these mice still maintained large populations of CAR T cells (green dots), an indication of CAR T cell persistence, and protection of the mouse from engraftment of REH tumor cells sequestered in other organs. To determine if this persistence could be due to secretion of IL15/IL-15sushi, the plasma from each mouse was subjected to ELISA to quantify the amount of secreted IL15. As shown in figure 44C, human IL-15 was not detected in the two control mice, but a concentration of 159 pg/mL was detected in the CAR T cell treated mouse that still appeared to be relatively healthy on Day 36, when it was euthanized.

Function of IL15 in CD20h-CD19b-IL15/IL-15sushi CAR NK cells.

To further determine if IL-15 is being secreted, NK-92 cell line was transduced with lentiviral vector containing CD20h-CD19b-IL15/IL-15sushi CAR. Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype (Figure 44D). Sorted cells were expanded, and after 72 hours supernatant was collected and subjected to ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained on a plate reader were compared to a standard curve generated with human IL-15 to determine concentration of IL-15 in the supernatant (Figure 44E). It was determined that IL-15 was detected in the supernatant at 328 pg/mL. By comparison, supernatant containing approximately the same number of wild-type control NK-92 cells had a background concentration of only 0.33 pg/mL.

IL15/IL15sushi secreted from CD20h-CD19b-IL15/IL15sushi CAR NK cells can substitute for the function of IL-2 in vitro related to expansion and growth.

Sorted CD20h-CD19b-IL15/IL15sushi CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5×10^6 cells per mL, in 1 mL total volume. Cells were added to duplicate wells; one well of each pair contained IL-2 at 300 IU/mL, the other well did not. After 48 hours (Day 2), cells were counted, and the volume increased to yield a concentration of approximately 0.5×10^6 cells/mL. This process was repeated on Days 4, 6 and 8. As shown in the graph in figure 44D, CD20h-CD19b-IL15/IL-15sushi NK CAR cells cultured for 8 days *without* IL-2 in the culture expanded at the same rate as wild-type NK-92 cells cultured *with* IL-2 added, whereas wild-type NK-92 cultured *without* IL-2 had all died by Day 6. This indicates that IL-15 secreted by the NK CAR cells can substitute for the expansion activity of IL-2.

In one embodiment, the engineered cell includes a CD20-CD19 cCAR polypeptide, and IL-15/IL-15sushi (SEQ ID NO. 28), and corresponding nucleotides (SEQ ID NO. 29).

In one embodiment, the engineered cell includes a CD20-CD19 cCAR polypeptide (SEQ ID NO. 52), and corresponding nucleotides (SEQ ID NO. 53).

5

Generation of CD19b-IL-15/IL-15sushi CAR

CD19b-IL-15/IL-15sushi CAR T-cells were generated by transduction of primary peripheral blood T-cells with the lentiviral construct as previously described (Pinz, 2015).

CD19b-IL-15/IL-15sushi CAR construct contains one unit of CAR, anti-CD19 CAR (also called CD19b CAR). CD19b-IL-15/IL-15sushi CAR is able to secrete IL-15/IL-15sushi (Figure 45A). The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion can enhance CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

Flow cytometry analysis showed that ~35% of T cells expressed the CD19b-IL-15/IL-15sushi CAR F(Ab')₂ fragment after transduction (Figure 45A). This CD19b-IL-15/IL-15sushi CAR was designed to: 1) delete targeted tumor cells, 2) enhance anti-tumor cytotoxicity, 3) robustly increase CAR potency and persistency by secreting the IL-15/IL-15sushi fusion.

Co-culture experiments were performed at an effector to target (E:T) ratio of spanning from 1:1 to 5:1 for 24 hours and were directly analyzed by flow cytometry with mouse anti-human CD3pPerCp and mouse anti-human CD19-PE. Each assay consists of target cells (Sp53 all CD19+) incubated with either P2A control or CAR T-cells. This experiment revealed the dose-dependent nature of the CD19b-IL-15/IL-15sushi CAR T cells, where even at low E:T ratios such as 1:1, there was potent lysis of tumor cells of greater than 60%. At 2:1, saturation of killing ability was observed with all tumor cells lysed (Figure 45B).

CD19b-IL-15/IL-15sushi CAR T-cells potently lyse CD19+ Sp53 cells (with comparison to CD19b single CART cells)

Similar cocultures conditions were used as above (Fig 45B), in this experimental scheme, anti-CD19 CAR co-expressing IL-15/IL-15sushi (CD19b-IL-15/IL-15sushi) CAR T cells were

cultured against CD19 positive Reh cells in comparison to both control P2A and single anti-CD19b CAR T cells. Anti-CD19b CART cells were generated with the same methodology and expression on T cell surfaces was verified to be ~50% (of all T cells, data not shown). The results here demonstrate that even at low E:T ratios such as 1:1, both CART treatments are
5 equally effective, with potent and virtual deletion of all antigen-positive Reh cells. The “secreting IL-15/IL-15sushi fusion” does not have a deleterious effect on the cytotoxicity of the CAR T cells.

CD19b-IL-15/IL-15sushi CART constructs provide enhanced persistency and biologic activity compared to standard CD19 CAR

10 To characterize the CAR secreting IL-15/IL-15sushi as a viable option to current CAR T /NK cell paradigms we analyzed for 3 broad factors: 1) ability to kill target cells (efficacy), 2) enhanced persistence for increased bioavailability and surveillance, and 3) proliferation of more potent CAR T phenotypes. We found that the CD19b-IL-15/IL-15sushi CAR construct was able to control Reh model tumor growth in *vivo* with comparable and slightly better efficacy than
15 standard CART-19 (CD19b CAR) (Figure 46A). In a second Reh model, we showed that as time goes on, Reh tumor relapsed in standard CAR T(CD19b CAR) treatment, however, the IL-15/IL-15sushi secreting CAR persists and deletes relapsed tumor and keeps mice disease free (Figure 46B and 46C). Furthermore, by survival endpoints, CD19b-IL-15/IL-15sushi CAR T cells administered mice revealed distinct populations of cytotoxic T cells remaining in circulation with
20 a higher level, compared to CD19b CAR without secreting IL-15/IL-15sushi (Figure 46D). In addition, the population of remaining T cells in both treatment groups showed that the CAR T cells (CD19b-IL-15/IL-15sushi) secreting IL-15/IL-15sushi resulted in a more cytotoxic T cell population, comprised of a higher population of CD8+ cells (Figure 46D and E).

Generation of BCMA-CD38 compound CARs

25 Three versions of compound CARs, CD269-A7D-CD38b, CD269-A7D-CD38a, and CD269-A7D-CD38c were created and their CAR T cells were generated by transduction of primary peripheral blood T-cells with the lentiviral construct as previously described (Pinz, 2015). Flow cytometry analysis showed various levels of CAR T cell expression with F(Ab')₂ fragment after transduction (Figure 45A).

30 CD269-A7D-CD38a or CD269-A7D-CD38b CAR T cells from Figure 45A were assayed for their ability to specifically lyse REH naturally expressing CD38. Co-cultures with control T

cells or CD269-A7D-CD38a or CD269-A7D-CD38b CAR T cells with REH wild-type cells were set up at 2:1 and 5:1 effector cell:target cell ratios, for 24 hours. Both CAR T cells demonstrated robustly lysed targeted cells (Figure 47B).

CD269-A7D-CD38a or CD269-A7D-CD38b CAR T cells were also tested for their ability of targeting K562 cells synthetically expressing BCMA. Co-cultures with control T cells or CD269-A7D-CD38a or CD269-A7D-CD38b with either wild type or K562 expressing BCMA (k-BCMA) cells were set up at 2:1 and 5:1 effector cell:target cell ratios, for 24 hours. Following this incubation, cells were stained using mouse anti-human CD3 antibody (in all cases), and either mouse anti-human BCMA (CD269), and analyzed by flow cytometry. Both CAR T cells showed remarkably lysed targeted cells (figure 47C).

Structural organization of CD269-A7D-CD38a-IL15/IL15sushi CAR (Also called BCMA-CD38-IL-15/IL-15sushi CAR)

CD269-A7D-CD38a-IL15/IL15sushi (Figure 48A) contains two independent units of two CARs, CD269-A7D (also called anti-BCMA CAR or anti-CD269 CAR), and CD38a CAR (also called anti-CD38). CD269-A7D-CD38a-IL15/IL15sushi is able to secrete IL-15/IL-15sushi. The soluble IL-15/IL-15sushi fusion is stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion can enhance CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

CAR expression

Activated human peripheral blood T cells were transduced with the lentiviral vector from CD269-A7D-CD38a-IL15/IL15sushi CAR. Figure 1 shows the transduction efficiency between activated T cells transduced with either control vector, or CD269-A7D-CD38a-IL15/IL15sushi CAR vector, as determined by labeling with goat anti-mouse F(Ab')₂ antibody. Activated T cells transduced with the CAR vectors resulted in 33% F(Ab')₂ positive cells for CD269-A7D-CD38a-IL15/IL15sushi (Figure 48B). These CAR T cells were used in the following in vitro killing assays.

T cells transduced with CD269-A7D-CD38a-IL15/IL15sushi CAR exhibit self-killing of CD38+ T cells

To determine if T cells transduced with CD269-A7D-CD38a-IL15/IL15sushi CAR would be lysed by the CD38a CAR domain, cells were harvested on Day 6 (the same day as anti-F(Ab')₂ labeling, above) and Day 12. Cells were labeled with mouse anti-human CD3 and CD38, and analyzed by flow cytometry. As shown in Figure 2, the number of CAR T cells on Day 6 expressing CD38 was cut nearly in half (97% to 51%) in comparison to the control T cells, while nearly all CAR cells by Day 12 were CD38-. This confirms the fratricide, or self-killing, of CD38+ T cells, by CD38a CAR.

CD269-A7D-CD38a-IL15/IL15sushi CAR T cells are able to lyse tumor cell lines expressing either BCMA (CD269) or CD38 antigens in vitro assays

CD269-A7D-CD38a-IL15/IL15sushi CAR T cells from Figure 48B were assayed for their ability to specifically lyse K562 cells synthetically expressing BCMA (CD269) antigen or wild-type REH cells, which naturally express CD38 antigen. Wild-type K562 cells were transduced with lentiviral vector for BCMA antigen expression and positively selected by FACS (FACS-Aria, BD) to create K-BCMAxp cell line. Co-cultures with control T cells or CD269-A7D-CD38a-IL15/IL15sushi CAR T cells, and either K-BCMAxp or REH cells were set up at 2:1 and 5:1 effector cell:target cell ratios, for 24 or 48 hours. Following this incubation, cells were stained using mouse anti-human CD3 antibody (in all cases), and either mouse anti-human CD269 or CD38, and analyzed by flow cytometry. The results demonstrated that each CAR component of the CD269-A7D-CD38a-IL15/IL15sushi CAR T cell was able to lyse its intended target cells (figure 48D).

Function of IL-15 in CD269-A7D-CD38a -IL15/IL15sushi CAR NK cells.

To determine if IL-15 is being secreted, NK-92 cell line was transduced with lentiviral vector containing CD269-A7D-CD38a-IL15/IL15sushi CAR. Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype (Figure 48E). Sorted cells were expanded, and after 72 hours supernatant was collected and subjected to ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained on a plate reader were compared to a standard curve generated with human IL-15 to determine concentration of IL-15 in the supernatant (Figure 48F). It was determined that IL-15 was detected in the supernatant at 512 pg/mL. By comparison, supernatant containing approximately the same number of wild-type control NK-92 cells had a background concentration of only 0.

IL15/IL15sushi secreted from CD269-A7D-CD38a -IL15/IL15sushi CAR NK cells can substitute for the function of IL-2 in vitro related to the expansion and growth

Sorted CD269-A7D-CD38a -IL15/IL15sushi CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5×10^6 cells per mL, in 1 mL total volume. Cells were added to duplicate wells; one well of each pair contained IL-2 at 300 IU/mL, the other well did not. After 48 hours (Day 2), cells were counted, and the volume increased to yield a concentration of approximately 0.5×10^6 cells/mL. This process was repeated on Days 4, 6 and 8. As shown in the graph in figure 48E, CD269-A7D-CD38a -IL15/IL15sushi NK CAR cells cultured for 8 days *without* IL-2 in the culture expanded at the same rate as wild-type NK-92 cells cultured *with* IL-2 added, whereas wild-type NK-92 cultured *without* IL-2 had all died by Day 6. This indicates that IL-15 secreted by the NK CAR cells can substitute for the expansion activity of IL-2.

CD123b-CD33b-IL15/IL15sushi cCAR

Structural organization of CD123b-CD33b-IL15/IL15sushi (also called CD123-CD33-IL-15/IL-15sushi) and CD123b-CLL1-IL15/IL15sushi (also called CD123-CLL1-IL15/IL-15sushi) cCARs

CD123b-CD33b-IL15/IL15sushi contains two independent units of two CARs, CD123b CAR (also called anti-CD123 CAR), and CD33b CAR (also called anti-CD33 CAR).

CD123b-CLL1-IL15/IL15sushi also contains two independent units of two CARs, CD123b CAR (also called anti-CD123 CAR), and CLL-1 CAR (also called anti-CLL1 CAR). Both CAR constructs were generated using a similar method described.

Both CARs were able to secrete IL-15/IL-15sushi. The soluble IL-15/IL-15sushi fusion are stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells or NK T cells, and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion can enhance CAR T/NK or NK T cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

Function of IL-15 in CD123b-CD33b-IL15/IL15sushi and CD123b-CLL1-IL15/IL15sushi CARs

To determine if IL-15 is being secreted, NK-92 cell line was transduced with lentiviral vector containing CD123b-CD33b-IL15/IL15sushi or CD123b-CLL1-IL15/IL15sushi. Cells were sorted on BD FACS Aria to select NK cells positive for the F(Ab')₂ phenotype (Figure 49B). Sorted cells were expanded, and after 72 hours supernatant was collected and subjected to ELISA on 96-well plates precoated with IL-15 antibody. Following manufacturer's (Boster) directions, colorimetric results obtained on a plate reader were compared to a standard curve generated with human IL-15/IL-15sushi to determine concentration of IL-15sushi in the supernatant (Figure 49C). It was determined that IL-15sushi was detected in the supernatant at >1500 pg/mL and >1000 pg/mL from CD123b-CD33b-IL-15/IL-15sushi and CD123b-CLL1-IL-15/IL-15sushi NK cells, respectively. By comparison, supernatant containing approximately the same number of wild-type control NK-92 cells had a background concentration close to 0.

IL15/IL15sushi secreted from CD123b-CD33b-IL15/IL15sushi and CD123b-CLL1-IL15/IL15sushi CAR NK cells can substitute for the function of IL-2 in vitro related to the expansion and growth

CD123b-CD33b-IL15/IL15sushi and CD123b-CLL1-IL15/IL15sushi
Sorted CD123b-CD33b-IL15/IL15sushi or CD123b-CLL1-IL15/IL15sushi CAR NK cells, and wild-type NK-92 cells, were cultured in a 24-well plate at 0.5×10^6 cells per mL, in 1 mL total volume. Cells were added to duplicate wells; one well of each pair contained IL-2 at 300 IU/mL, the other well did not. After 48 hours (Day 2), cells were counted, and the volume increased to yield a concentration of approximately 0.5×10^6 cells/mL. This process was repeated on Days 4, 6 and 8. As shown in the graph in figure 49B, CD123b-CD33b-IL15/IL15sushi or CD123b-CLL1-IL15/IL15sushi NK CAR cells cultured for 8 days *without* IL-2 in the culture expanded at the same rate as wild-type NK-92 cells cultured *with* IL-2 added, whereas wild-type NK-92 cultured *without* IL-2 had all died by Day 8. This indicates that IL-15 secreted by the NK CAR cells can substitute for the expansion activity of IL-2.

Examples for generation of UCAR (universal CAR)

Generation of BCMA15/IL-15sushi-CAR expressed human NK cells prepared from human cord blood

BCMA15/IL-15sushi-CAR construct (also called CD269-A7D-IL-15/IL-15sushi or BCMA CARVac) contains one unit of CAR, CD269-A7D (also called anti-BCMA CAR or anti-CD269 CAR) (Figure 50A). BCMA15/IL-15sushi-CAR is able to secrete IL-15/IL-15sushi. The soluble IL-15/IL-15sushi fusion is stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion can enhance CAR T/NK cell persistency, stimulate tumor infiltrate lymphocyte proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

Generation of feeder cells for expansion of cord blood NK cells:

The steps for generation of feeder cells are shown in Figure 50B with a flowchart. K562 cells are transduced with lentiviruses expressing a surface anchor protein or scFv tagged IL-21 (IL-21 anchor) (see Figure 55) or scFv tagged 4-1BBL and IL-15/IL-15sushi anchor (also called super 2)(Figure 54 and Figure 55).

In one embodiment, the engineered K562 cell includes IL-21 anchor polypeptide (SEQ ID NO. 1), and corresponding nucleotides (SEQ ID NO. 2).

In one embodiment, the engineered K562 cell includes super2 polypeptide (SEQ ID NO. 50), and corresponding nucleotides (SEQ ID NO. 51).

K562 were transduced with IL-21 anchor or super 2 lentiviruses for 48 hours. After transduction, cells are expanded and labeled by antibodies for sorting of genetically modified K562 cells by FACS. Sorted genetically modified K562 cells are expanded, irradiated (10-100Gy) and frozen down until use. Irradiated genetically modified K562 cells are added into cord blood cell to stimulate and expand NK cells as feeder cells.

Expansion of human NK cells from human cord blood (Figure 50C).

Flowchart (Figure 50C) shows the steps for generation and expansion of CAR-transduced natural killer (NK) cells from umbilical cord blood by co-culture with irradiated genetically modified K562 cells. Cord blood cells are suspended into T-cell culture mediums with 300U/ml IL-2 for 48 h. Irradiated genetically modified K562 cells are added into cord blood cell to stimulate and expand NK cells for 48h.

Stimulated cord blood cells are transduced with CAR lentiviruses and incubated for up to 48h. CAR transduced cord blood cells are co-cultured with irradiated genetically modified K562 feeder cells again.

Every 2 to 3 days, CAR transduced cord blood cells are counted and fed with fresh
5 mediums to maintain cell condition. Exogenous IL-2 is added in all of cell culture medium.

After 2 weeks, fold expansion of NK cells become 220-680 times compared to first day. After 3 weeks, fold expansion of NK cells become 450-1500 times compared to first day.

The percentage of NK cells and T-cells, and expression of CAR on NK cells are determined by flow cytometry analysis using antibodies against murine Fab fragment.

10 Flow cytometry analysis showed the expression levels of BCMA-IL15/IL15sushi-CAR on CD56 positive cells (blue dots circled in pink) in cord blood cells after transduction BCMA-IL15/IL15sushi-CAR- viruses in cord blood cells.

These data indicate that transduction of BCMA-IL15/IL15sushi-CAR-viruses into cord blood NK cells successfully generate BCMA-IL15/IL15sushi-CAR-expressed NK cells.

15 **BCMA-IL-15/IL15sushi-CAR NK cells demonstrate remarkably and unexpected anti-leukemic effects *in vivo* mouse model.**

In order to evaluate the specific *in vivo* anti-tumor activity of BCMA-IL-15/IL-15sushi-CAR expressed NK cells derived human cord blood, we developed a xenogeneic mouse model using NSG mice sublethally irradiated and intravenously injected with luciferase-expressing
20 MM1S multiple myeloma cells to induce measurable tumor formation. At day 4, the mice were intravenously injected with 10×10^6 control NK cells or BCMA-IL15/IL15sushi-CAR expressed NK cells. On days 3, 6, 8 and 10, mice were injected subcutaneously with RediJect D-Luciferin and subjected to IVIS imaging. As observed by IVIS imaging, total flux levels continually increased in control mice with tumor burden growth. In contrast, BCMA-IL15/IL15sushi-CAR-
25 NK cells injected mice significantly suppressed tumor burden as early as day 6 with a 59.5% reduction in tumor burden. Results showed that only 10 days following treatment with CAR NK cells (Day 6), mice treated with BCMA-IL-15/IL-15sushi CAR NK cells had >84% lower tumor burden than mice given control NK cells (Figure 51A and 51B). In addition, CAR NK cell-treated mice also had very significantly more favorable survival outcomes. A survival curve was
30 generated to show the survival of CAR NK cell-treated mice over time, more than 80 days, with

Log-rank (Mantel-Cox) Test $p = 0.0069$ as compared to controls, which only survived up to 45 days (Figure 51C).

Evaluation of persistence of infused BCMA-A7D-IL15/IL15sushi CAR transduced NK cells in vivo.

5 In order to evaluate the persistence of BCMA-A-7D-IL-15/IL-15sushi (also called CD269-IL-15/IL-15sushi) NK cells, , we developed a xenogeneic mouse model using NSG mice sub-lethally irradiated and intravenously injected with 1×10^6 of luciferase-expressing MM1S multiple myeloma cells to induce measurable tumor formation. On Day 4, leukemic mice were intravenously injected with 10×10^6 BCMA-A-7D-IL-15/IL-15sushi NK cells derived human
10 cord blood. Evaluation of persistence of infused BCMA-A7D-IL15/IL15sushi CAR transduced NK cells in xenograft mouse model were done *on Day 25* (Figure 52A) *and Day 60* (Figure 52B). On Day 25 (21 days after control NK or CAR NK cells infused mice) and Day 60 (58 days after mice were infused with control NK or CAR NK cells), peripheral blood was collected from individual mice and cells were labeled using human CD56-and human CD45 antibodies to detect
15 the presence of infused control- and/or CAR- NK cells. MM1S myeloma cell line is negative for CD56 and CD45 (Figure 52A left panel), which can be used to monitor human NK cells in mice. The persistence of control NK cells or BCMA-IL15/IL15sushi CAR transduced NK cells in collected peripheral blood was determined by flow cytometry analysis. Left panels show that MM1S was negative for CD56 and CD45. BCMA-IL15/IL15sushi-CAR transduced NK cells
20 persisted more than 25 days (Figure 52A) and 60 days (Figure 52B) after infusion, while non-transduced NK cells (control) were un-detectable in mice. In addition, further evaluation showed that BCMA-IL15/IL15sushi-CAR transduced NK cells could persist more than three months. In general, human non-transduced NK cells usually persist less than one or two weeks in mice.

25 The natural killer cell is an ideal platform for creating a universal CAR that avoids risks associated with genome editing. However, the life expectancy of NK CAR cells *in vivo* is very short, with a lifespan of one or two weeks. Ideally, the NK cell persistency should be one or two months to be considered adequate for therapy. We have developed a NK cell platform for a universal CAR therapy with improved persistency and killing using IL-15/IL-15sushi fusion. The invented studies demonstrate CAR NK cells co-expressing secretory IL-15/IL-15sushi can be
30 used as non-gene-editing universal CAR platform for treatment of a variety of diseases.

CD123 CAR super1**Structural organization of CD123 CAR super 1**

CD123 CAR super 1 (Figure 57) contains a CAR, CD123 CAR (also called anti-CD123 CAR or CD123b CAR). CD123b CAR super 1 also co-expresses 4-1BBL ligand to enhance
5 CAR function, and a secreting IL-15/IL-15sushi fusion. The soluble IL-15/IL-15sushi fusion is stable and functions as an unexpected and powerful immunomodulatory for CAR T/NK or NK T cells and their neighbor tumor immune response cells. The soluble IL-15/IL-15sushi fusion can enhance CAR T/NK or NK T cell persistency, stimulate tumor infiltrate lymphocyte
10 proliferation, and anti-tumor activity. The soluble IL-15/IL-15sushi fusion provides anti-tumor vaccine-like effects by reprogramming body's immune system to fight cancers.

CAR Expression

NK cells from cord blood were transduced with the lentiviral vector expressing CD123b CAR super 1. Figure 58 shows the transduction efficiency between NK cells transduced with either control vector, or CD123b super 1, as determined by labeling with goat anti-mouse
15 F(Ab')₂ antibody. Activated T cells transduced with the CAR vectors resulted in 8% F(Ab')₂ positive cells for CD123b super 1(Figure 58). These CAR T cells were used in the following in vitro killing assays. The functional testing will be performed as described above in vitro and in vivo.

In one embodiment, the engineered cell includes a CD123b chimeric antigen receptor
20 polypeptide and 4-1BBL ligand, and IL-15/IL-15sushi (SEQ ID NO. 32), and corresponding nucleotides (SEQ ID NO. 33).

BCMA-CD38a-IL15/IL15sushi cCAR in NK cells derived from human cord blood

25 NK cells from cord blood were transduced with the lentiviral vector expressing BCMA-CD38a-IL15/IL15sushi cCAR. Figure 59 shows the transduction efficiency between NK cells transduced with either control vector, or BCMA-CD38a-IL15/IL15sushi cCAR, as determined by labeling with goat anti-mouse F(Ab')₂ antibody. Activated T cells transduced with the CAR vectors resulted in 40% F(Ab')₂ positive cells for BCMA-CD38a-IL15/IL15sushi cCAR (Figure
30 59). These CAR T cells were used in the following in vitro killing assays. The functional testing will be performed as described above in vitro and in vivo.

In one embodiment, the engineered cell includes BCMA-CD38a chimeric antigen receptor polypeptides and 4-1BBL ligand, and IL-15/IL-15sushi (SEQ ID NO. 40), and corresponding nucleotides (SEQ ID NO. 41).

5

GD2-Super1-CAR

EXAMPLE

The structural organization of GD2 super1 CAR shown in Figure 60A. Links by P2A and T2A schematic to generate a super1 CAR showing a CAR, GD2 CAR equipped with 4-1BBL and IL-15/IL-15sushi in a single construct. The construct consists of a SFFV promoter driving
10 the expression of three segments, CAR, 4-1BBL and IL-15/IL-15sushi. Upon cleavage of the linkers (P2A and T2A), the CAR, 4-1BBL and IL-15/IL-15sushi split and engage upon a target (s). CAR has scFV, hinge region, transmembrane domain, costimulatory domain (including, but not limited to, CD28 or 4-1BB) and intracellular signaling, CD3 zeta chain. 4-1BBL or IL-15/IL-sushi or both provides a synergistic effect of T or NK cell activation and persistency or
15 anti-tumor activity with CD28 or 4-1BB.

In order to evaluate the in vivo anti-tumor activity of various GD2-targeting CAR constructs, we developed a xenogeneic mouse model using NSG mice sublethally irradiated and intravenously injected with luciferase-expressing Y79 retinoblastoma cells to induce measurable
20 tumor formation. Three days following tumor cell injection, mice were intravenously injected with a course of 10×10^6 of either GD2-CAR, GD2-4-1BBL CAR, or GD2-super1 CAR, or vector control T cells. To determine the persistence of CAR T cells, mice were euthanized on Day 30. Liver, spleen and whole blood was collected from each mouse.

Flow cytometry analysis shows persistence of Y79 tumor (blue dots) in the livers of mice
25 treated with different forms of anti-GD2 CAR T cells (Figure 60B). Homogenized liver cells were labeled with mouse anti-human CD3 and CD56 antibodies, to detect human T cells and Y79 tumor cells, respectively. A representation of a mouse given control T cells is shown on the left; mouse treated with GD2CAR (left center), GD2-4-1BBL CAR (right center), and GD2-super1 CAR (right) T cells. Figure 60B shows that GD2CAR T cells were unable to eliminate
30 Y79 cells from the liver, relative to the mouse given control T cells, while mice treated with GD2-4-1-BBL CAR T cells had 32% fewer tumor cells. By contrast, the GD2-super1 CAR

treated mice had 85% less tumor cells in the liver. A graph was then constructed to indicate percent killing activity against Y79 cells by each CAR treated mice compared to control mice (n=2) (Fig. 60B). From these data, especially, GD2-Super CAR eliminates Y79 cells in liver. Analysis of mice spleen showed a 1.87-fold increase in human T cells in GD2-super1 treated mice compared to control mice (Figure 60C), and higher than GD2CAR (1.15x) and GD2-4-1BBL (1.35). This increase in GD2-super1 T cells is even more pronounced in the analysis of mouse whole blood, where there is a nearly 3-fold increase over control mice, and more than double the percentage of GD2CAR (Figure 60D). A graph was then created to indicate the persistence of human T cells in whole blood samples, relative to the number of total cells analyzed by flow cytometry (n= 2 each) (Figure 60E). These data strongly suggest that GD2-super1 CAR, with both secreted IL-15/IL-15sushi and 4-1BBL domains, lyses GD2-expressing tumor cells and exhibits greater persistence than GD2CAR or GD2-41BBL CAR T cells.

In one embodiment, the engineered cell includes GD2 chimeric antigen receptor polypeptides and 4-1BBL ligand (SEQ ID NO. 58), and corresponding nucleotides (SEQ ID NO. 59).

In one embodiment, the engineered cell includes GD2 chimeric antigen receptor polypeptides SEQ ID NO. 56), and corresponding nucleotides (SEQ ID NO. 57).

In one embodiment, the engineered cell includes GD2 chimeric antigen receptor polypeptides, 4-1BBL ligand and IL-15/IL-15sushi (SEQ ID NO. 58), and corresponding nucleotides (SEQ ID NO. 59).

DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO.	DESCRIPTION
SEQ ID NO: 1	CD19b-IL-21 anchor CAR amino acid sequence
SEQ ID NO: 2	CD19b-IL-21 anchor CAR nucleotide sequence
SEQ ID NO: 3	CD269-A7D-C11D cCAR, also called Ab269-7-11 CAR amino acid sequences
SEQ ID NO: 4	CD269-A7D-C11D cCAR, also called Ab269-7-11 CAR nucleotide sequence
SEQ ID NO: 5	CD269-7D-CD38-3077-2G amino acid
SEQ ID NO: 6	CD269-7D-CD38-3077-2G CAR nucleotide sequence

SEQ ID NO: 7	CD269-A7D-CD38-3079 CAR amino acid sequence
SEQ ID NO: 8	CD269-A7D-CD38-3079 CAR nucleotide sequence
SEQ ID NO: 9	CD269-A7D-CD38 CAR amino acid sequence
SEQ ID NO: 10	CD269-A7D-CD38 CAR nucleotide sequence
SEQ ID NO: 11	BCMA-A7D-IL-18 CAR amino acid sequence
SEQ ID NO: 12	BCMA-A7D-IL-18 CAR nucleotide sequence
SEQ ID NO: 13	BCMA-A7D-IL-18 anchor CAR amino acid sequence
SEQ ID NO: 14	BCMA-A7D-IL-18 anchor CAR nucleotide sequence
SEQ ID NO: 15	CD38 extracellular domain, CD38 XP
SEQ ID NO: 16	CD19b-IL-21 CAR amino acid sequence
SEQ ID NO: 17	CD19b-IL-21 CAR nucleotide sequence
SEQ ID NO: 18	CD3 -28-super1 CAR amino acid sequence
SEQ ID NO: 19	CD3 -28-super1 CAR nucleotide sequence
SEQ ID NO: 20	CD4 -28-super1-2G CAR amino acid sequence
SEQ ID NO: 21	CD4 -28-super1-2G CAR nucleotide sequence
SEQ ID NO: 22	CD4-IL15/IL15sushi-3G CAR amino acid sequence
SEQ ID NO: 23	CD4-IL15/IL15sushi-3G CAR nucleotide sequence
SEQ ID NO: 24	CD19b-28-IL-15/IL15sushi-2G CAR amino acid sequence
SEQ ID NO: 25	CD19b-28-IL-15/IL15sushi-2G CAR nucleotide sequence
SEQ ID NO: 26	CD19b-28-super1-2G CAR amino acid sequence
SEQ ID NO: 27	CD19b-28-super1-2G CAR nucleotide sequence
SEQ ID NO: 28	CD20hCD19b -IL15/IL15sushi-2G CAR amino acid sequence
SEQ ID NO: 29	CD20hCD19b -IL15/IL15sushi-2G CAR nucleotide sequence
SEQ ID NO: 30	CD33b -28-super1-2G CAR amino acid sequence
SEQ ID NO: 31	CD33b -28-super1-2G CAR nucleotide sequence
SEQ ID NO: 32	CD123b-28-super1-2G CAR amino acid sequence
SEQ ID NO: 33	CD123b -28-super1 CAR nucleotide sequence
SEQ ID NO: 34	CD123b-CD33b-IL15/IL15sushi-2G CAR amino acid sequence

SEQ ID NO: 35	CD123b-CD33b-IL15/IL15sushi-2G CAR nucleotide sequence
SEQ ID NO: 36	CD123b-CLL1-28-IL/IL15sushi-2G CAR amino acid sequence
SEQ ID NO: 37	CD123b-CLL1-28-IL/IL15sushi-2G CAR nucleotide sequence
SEQ ID NO: 38	CD269-A7D-28-super1-2G CAR amino acid sequence
SEQ ID NO: 39	CD269-A7D-28-super1-2G CAR nucleotide sequence
SEQ ID NO: 40	CD269-A7D-CD38a -IL15/IL15sushi-2G CAR amino acid sequence
SEQ ID NO: 41	CD269-A7D-CD38a -IL15/IL15sushi-2G CAR nucleotide sequence
SEQ ID NO: 42	CD269-A7D-CS1-hu63-28-IL15/IL15sushi-2G CAR amino acid sequence
SEQ ID NO: 43	CD269-A7D-CS1-hu63-28-IL15/IL15sushi-2G CAR nucleotide sequence
SEQ ID NO: 44	CLL1-28-super1-2G CAR amino acid sequence
SEQ ID NO: 45	CLL1-28-super1-2G nucleotide sequence CAR nucleotide sequence
SEQ ID NO: 46	GD2-28-super1-2G CAR amino acid sequence
SEQ ID NO: 47	GD2-28-super1-2G CAR nucleotide sequence
SEQ ID NO: 48	L45-CD5-28-52-IL-15/IL-15sushi-2G CAR amino acid sequence
SEQ ID NO: 49	L45-CD5-28-52-IL15/IL-15sushi-2G CAR nucleotide sequence
SEQ ID NO: 50	CD269-A7D-super2-2G CAR amino acid sequence
SEQ ID NO: 51	CD269-A7D-super2-2G CAR nucleotide sequence
SEQ ID NO: 52	D20h-CD19b-28-2G CAR amino acid sequence
SEQ ID NO: 53	CD20hCD19b-28-2G CAR nucleotide sequence
SEQ ID NO: 54	CD45b-28-2G-IL-15/IL-15sushi amino acid sequence
SEQ ID NO: 55	CD45b-28-2G-IL-15/IL-15sushi nucleotide sequence
SEQ ID NO: 56	GD2-28-2G CAR amino acid sequence
SEQ ID NO: 57	GD2-28-2G CAR nucleotide sequence
SEQ ID NO: 58	GD2-28-4-1BBL-2G CAR amino acid sequence

SEQ ID NO: 59	GD2-28-41BBL-2G CAR nucleotide sequence
SEQ ID NO: 60	CLL1-CD33b-IL15/IL15sushi-2G CAR amino acid sequence
SEQ ID NO: 61	CLL1-CD33b-IL15/IL15sushi-2G CAR nucleotide sequence

CLAIMS

1. An ex vivo engineered T cell or NK cell co-expressing two distinct chimeric antigen receptor (CAR) units at the cell surface, wherein the engineered T cell or NK cell comprises a nucleotide sequence comprising from 5' to 3' a first polynucleotide encoding a first chimeric antigen receptor polypeptide (first CAR), a second polynucleotide encoding a second chimeric antigen receptor polypeptide (second CAR), a nucleotide encoding a first viral self-cleavage peptide disposed between the first CAR and second CAR, under the transcriptional control of a single promoter, wherein:
 - (i.) the first CAR polypeptide comprises a first antigen recognition domain selected from the group consisting of CS-1, CD5, CD19, CD20, CD123, BCMA, CD38, CLL-1, and CD33; a first signal peptide; a first hinge region; a first transmembrane domain; a first co-stimulatory domain; and a first signaling domain; and
 - (ii.) the second CAR comprises a second antigen recognition domain selected from the group consisting of CS-1, CD5, CD19, CD20, CD123, BCMA, CD38, CLL-1, and CD33; a second signal peptide; a second hinge region; a second transmembrane domain; a second co-stimulatory domain; and a second signaling domain;
 wherein the first antigen recognition domain and the second antigen recognition domain are different;

wherein the engineered T cell or NK cell comprises an enhancer selected from the group consisting of IL-15/IL-15sushi, IL-15/IL-15 sushi anchor, 4-1BBL, and IL-15; and

wherein the engineered T cell or NK cell comprises SEQ ID NO. 42 (a BCMA-CS 1 compound chimeric antigen receptor (cCAR) polypeptide and IL-15/IL- 15 sushi); SEQ ID NO. 34 (a CD123- CD33 cCAR polypeptide and IL-15/IL- 15 sushi); SEQ ID NO. 60 (a CLL1-CD33 cCAR polypeptide and IL-15/IL-15sushi); SEQ ID NO.40 (a BCMA-CD38 cCAR polypeptide, 4- 1BBL and IL-15/IL-15sushi); SEQ ID NO. 36 (CD123- CLL1 cCAR polypeptide, and IL-15/IL-15sushi); SEQ ID NO. 28 (a CD20-CD19 cCAR polypeptide, and IL-15/IL-15sushi; or SEQ ID NO. 50 (CD269-A7D- IL-15/IL-15sushi-2G), and wherein said enhancer is separated from the first CAR and second CAR by a nucleotide encoding a second viral self-cleavage peptide that flanks either end of the two distinct CAR units.

2. The engineered cell according to claim 1, wherein the enhancer is secreted by the engineered cell.
3. The engineered cell according to claim 1 or 2, wherein the engineered cell is an NK T cell, T cell, or NK cell.
4. The engineered cell according to any one of claims 1-3, wherein the engineered cell comprises at least two enhancers.
5. Use of an engineered cell according to claim 3 in the manufacture of a medicament for treating a cell proliferative disease, wherein the medicament is to be administered to a patient in need thereof, and wherein the cell proliferative disease comprises a T-cell malignancy, leukemia, or a lymphoma.
6. The use according to claim 5, wherein the cell proliferative disease comprises a soft tissue tumor.
7. The use according to claim 5 or 6, wherein the soft tissue tumor is selected from the group consisting of brain tumor, lung cancer, or osteosarcoma.