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(54) **Title**: COMPOSITIONS AND METHODS FOR INHIBITION OF LINEAGE SPECIFIC ANTIGENS

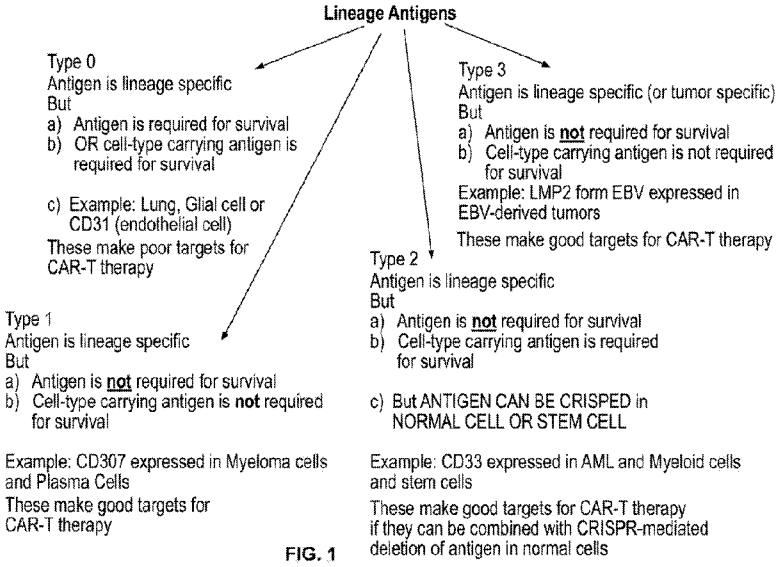


FIG. 1

(57) **Abstract**: Disclosed herein are methods of administering an agent targeting a lineage-specific cell-surface antigen and a population of hematopoietic cells that are deficient in the lineage-specific cell-surface antigen for immunotherapy of hematological malignancies.

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COMPOSITIONS AND METHODS FOR INHIBITION OF LINEAGE SPECIFIC ANTIGENS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Number 62/242,685, filed October 16, 2015, under U.S.C. §119, the entire content of which is herein incorporated by reference.

BACKGROUND OF DISCLOSURE

Despite decades of attempts, curative immunological therapy against cancer has been very difficult to achieve, with the fundamental basis being antigen-recognition capacity, either by antibodies or through T cells (via the T cell receptor) (Cousin-Frankel, *Science* (2013) 342:1432). Antibody-based immunotherapies have been used extensively against cancer in instances where the target antigen is up-regulated in tumor cells as compared to normal cells (*e.g.*, Her-2 in Her-2 amplified breast cancer), or in cases where the tumor cells express an antigen that can be recognized by the antibody or an antibody-toxin conjugate (*e.g.*, Rituximab against CD20) (Baselga et al., *Annals Oncology* (2001) 12:S35). While clinical trials using antibody-based immunotherapies have shown improved patient survival in a limited number of cancer types (usually when combined with standard chemotherapy), these effects are often accompanied by significant safety and efficacy concerns (Cousin-Frankel Cancer, *Science* (2013) 342:1432).

Effective T cell therapies against cancers have been even more difficult to achieve clinically (Schmitt et al., *Hum. Gene Ther.* (2009) 20(11):1240). An effective T cell therapy against cancer relies on a T cell with a high affinity binding directed against an antigen on a cancer cell. Chimeric antigen receptor T cells (CAR T cells) are widely used to recognize antigens on cells with both high affinity and specificity and without the requirement for accessory recognition molecules, such as HLA antigens to "present" peptides. The T cell receptor of a CAR T cells is "swapped" with an antigen-binding heavy and light chains, thereby obviating the need for HLA accessory molecules. The recombinant CAR T receptor is fused to signaling domains leading to activation of the T cell upon binding of the CAR T receptor to the target antigen.

The clinical use of CAR T cells has been limited to targeting a narrow range of cell surface antigens, further supporting the need for improved and novel approaches in the treatment of cancer. In particular, new approaches are needed for diseases such as acute

myeloid leukemia (AML) in which the outcomes in older patients who are unable to receive intensive chemotherapy, the current standard of care, remains very poor, with a median survival of only 5 to 10 months (Dohner et al., *NEJM* (2015) 373:1136).

Described herein are novel approaches to cancer immunotherapy that targets certain classes of lineage-specific cell-surface antigens on tumor cells. The CAR T cell treatment is then combined with replacement of the non-tumor cells by infusion or reinfusion of a modified population of cells that are deficient for the lineage-specific cell-surface antigen. Recurrence of the tumor is prevented or decreased by maintaining surveillance of the patient *in vivo* with the CAR T cells.

SUMMARY OF DISCLOSURE

The present disclosure is based, at least in part, on the discovery that agents comprising an antigen-binding fragment that binds a lineage-specific cell-surface antigen (*e.g.*, immune cells expression a chimeric receptor that targets CD33) selectively cause cell death of cells expressing the lineage-specific cell-surface antigen, whereas cells that are deficient for the antigen (*e.g.*, genetically engineered hematopoietic cells) evade cell death caused thereby. Based on such findings, it would have been expected that immunotherapies involving the combination of an agent targeting a lineage-specific cell-surface antigen, for example, CAR-T cells targeting CD33, and hematopoietic cells that are deficient in the lineage-specific cell-surface antigens (*e.g.*, CD33) would provide an efficacious method of treatment for hematopoietic malignancies.

One aspect of the present disclosure provides methods for treating a hematopoietic malignancy, the method comprising administering to a subject in need thereof (i) an effective amount of an agent targeting a lineage-specific cell-surface antigen, wherein the agent comprises an antigen-binding fragment that binds the lineage-specific cell-surface antigen; and (ii) a population of hematopoietic cells that are deficient in the lineage-specific cell-surface antigen. In some embodiments, the agent can be an immune cell (*e.g.*, a T cell) expressing a chimeric receptor that comprises the antigen-binding fragment that binds the lineage-specific cell-surface antigen. In some embodiments, the immune cells, the hematopoietic cells, or both, are allogeneic or autologous. In some embodiments, the hematopoietic cells are hematopoietic stem cells (*e.g.*, $CD34^+/CD33^-$ HSCs). In some embodiments, the hematopoietic stem cells can be obtained from bone marrow cells or peripheral blood mononuclear cells (PBMCs).

In some embodiments, the antigen-binding fragment binds a lineage-specific cell-surface antigen that is a type 2 lineage-specific cell-surface antigen (*e.g.*, CD33). In some embodiments, the antigen-binding fragment binds a lineage-specific cell-surface antigen that is a type 1 lineage-specific cell-surface antigen (*e.g.*, CD19).

In some embodiments, the antigen-binding fragment in the chimeric receptor is a single-chain antibody fragment (scFv) that specifically binds the lineage-specific cell-surface antigen, which can be a human protein, such as human CD33 or CD19. In some embodiments, the scFv binds to human CD33 and comprises a heavy chain variable region, which has the same complementary determining regions (CDRs) as those in SEQ ID NO:12, and a light chain variable region, which has the same CDRs as those in SEQ ID NO:13. In one example, the scFv comprises a heavy chain variable domain having the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain having the amino acid sequence of SEQ ID NO: 13.

The chimeric receptors may further comprise (a) a hinge domain, (b) a transmembrane domain, (c) at least one co-stimulatory domain, (d) a cytoplasmic signaling domain, or (e) a combination thereof. In some embodiments, the chimeric receptor comprises at least one co-stimulatory signaling domain, which can be derived from a co-stimulatory receptor of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen- 1 (LFA- 1), CD2, CD7, LIGHT, NKG2C, B7-H3, GITR, HVEM, or a combination thereof. In some embodiments, the at least one co-stimulatory signaling domain is a hybrid co-stimulatory domain comprising a signaling domain of CD28 and a signaling domain of ICOS. In one example, the at least one co-stimulatory signaling domain is from CD28 and the chimeric receptor further comprises a second co-stimulatory signaling domain from 4-1BB or ICOS.

In some embodiments, the chimeric receptor comprises a cytoplasmic signaling domain, which is from CD3ζ. In some embodiments, the chimeric receptor comprises a hinge domain, which is from CD8α or CD28α. In some embodiments, the chimeric receptor comprises a transmembrane domain, which is from CD8, CD28, or ICOS.

In some embodiments, the chimeric receptor comprises, from N terminus to C terminus, (i) a scFv that binds to the lineage-specific cell-surface antigen (*e.g.*, CD33 or CD19), a hinge domain from CD8α, a transmembrane domain from CD8, a costimulatory domain from 4-1BB, and a cytoplasmic signaling domain from CD3ζ; (ii) a scFv that binds to the lineage-specific cell-surface antigen (*e.g.*, CD33 or CD19), a hinge domain from CD8α, a

transmembrane domain from CD28, a costimulatory domain from CD28, and a cytoplasmic signaling domain from CD3ζ; or (iii) a scFv that binds to the lineage-specific cell-surface antigen (*e.g.*, CD33 or CD19), a hinge domain from CD8α, a transmembrane domain from CD28, a first costimulatory domain from CD28, a second costimulatory domain from 4-1BB, and a cytoplasmic signaling domain from CD3ζ.

In any of the methods described herein, the subject may have Hodgkin's lymphoma, non-Hodgkin's lymphoma, leukemia, or multiple myeloma. In some examples, the subject has leukemia, which is acute myeloid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, or chronic lymphoblastic leukemia.

In another aspect, the present disclosure provides a nucleic acid comprising a nucleotide sequence encoding any of the chimeric receptors as described herein. The chimeric receptor may comprise an antigen-binding fragment that binds CD33, a transmembrane domain, and a cytoplasmic signaling domain, such as a cytoplasmic signaling domain from CD3ζ. The antigen-binding fragment (*e.g.*, a scFv fragment) comprises a heavy chain variable region having the same CDRs as those in SEQ ID NO: 12, and a light chain variable region having the same CDRs as those in SEQ ID NO: 13. In some embodiments, the scFv comprises a heavy chain variable domain having the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain having the amino acid sequence of SEQ ID NO: 13.

Other aspects of the present disclosure provide vectors comprising any of the nucleic acids provided herein. Also within the scope of the present disclosure are chimeric receptors encoded by the nucleic acids described herein and immune cells (*e.g.*, T cells) expressing such a chimeric receptor. In some embodiments, the immune cells can be obtained from a patient having a hematopoietic malignancy.

Another aspect of the present disclosure provides genetically engineered hematopoietic cells (*e.g.*, HSCs) that are deficient in a lineage-specific cell-surface antigen (*e.g.*, CD33, CD19), which presents on the hematopoietic cell before genetic engineering. In some embodiments, the whole or a portion of an endogenous gene encoding the lineage-specific cell-surface antigen is deleted, for example by genome editing (*e.g.*, involving a zinc finger nuclease (ZFN), a transcription activator-like effector-based nuclease (TALEN), or a CRISPR-Cas system). In some embodiments, the lineage-specific cell-surface antigen is CD33 and a portion of the immunoglobulin constant (IgC) domain of the CD33 is deleted.

In some embodiments, the hematopoietic cell is a hematopoietic stem cell (*e.g.*, $CD34^+/CD33^-$ cell).

In some embodiments, the hematopoietic stem cells can be obtained from bone marrow cells or peripheral blood mononuclear cells (PBMCs).

Also provided herein are methods of producing a cell that is deficient in a lineage-specific cell-surface antigen as described herein. The methods comprises providing a cell, and introducing into the cell (i) a nucleic acid that comprises a nucleotide sequence of a CRISPR-Cas system guide RNA (gRNA), which hybridizes to a portion of the nucleotide sequence that encodes the lineage-specific cell-surface antigen (*e.g.*, CD33, CD19), and (ii) a Cas endonuclease (*e.g.*, Cas9 or Cpf1). The nucleic acid that comprises a nucleotides sequence of a CRISPR-Cas system gRNA and a Cas nuclease may, for example, be encoded on the same nucleic acid or on different nucleic acids, or introduced into the cell as a pre-formed ribonucleoprotein complex. In some embodiments, the portion of the nucleotide sequence to which the gRNA hybridizes consists of 18-22 nucleotides. In some examples, the gRNA comprises the nucleotide sequence of SEQ ID NO: 11 or 28-31.

In some embodiments, the cell is a hematopoietic cell, such as a hematopoietic stem cell (*e.g.*, $CD34^+$).

Also within the scope of the present disclosure are kits comprising (i) an immune cell of claim B13 an agent that target a lineage-specific, cell-surface antigen, which comprises an antigen binding fragment that binds the lineage-specific cell-surface antigen; and (ii) a population of hematopoietic cells (*e.g.*, hematopoietic stem cells) that are deficient in the lineage-specific cell-surface antigen. In some embodiments, the agent that targets the lineage-specific, cell-surface antigen is an immune cell expressing a chimeric receptor, which comprises the antigen-binding fragment the lineage-specific cell-surface antigen.

Further, the present disclosure provides pharmaceutical compositions comprising any of the immune cells targeting a lineage-specific cell-surface antigen and/or any of the hematopoietic cells that are deficient in the lineage-specific cell-surface antigen for use in treating a hematopoietic malignancy; as well as uses of the immune cells and hematopoietic cells for manufacturing a medicament for use in treating a hematopoietic malignancy.

The details of one of more embodiments of the disclosure are set forth in the description below. Other features or advantages of the present disclosure will be apparent from the detailed description of several embodiments and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIGURE 1 presents an exemplary illustration of type 0, type 1, type 2, and type 3 lineage-specific antigens.

FIGURE 2 is a schematic showing an immune cell expressing a chimeric receptor that targets the type 0 lineage-specific cell-surface antigen, CD307. Multiple myeloma (MM) cells expressing CD307 as well other cells expressing CD307, such as plasma cells, are targeted by the immune cells expressing the anti-CD307chimeric receptor.

FIGURE 3 is a schematic showing an immune cell expressing a chimeric receptor that targets the type 2 lineage-specific cell-surface antigen, CD33. Acute myeloid leukemia (AML) cells expressing CD33. Human hematopoietic stem cells (HSC) are genetically engineered to be deficient in CD33 and therefore not recognized by the immune cells expressing the anti-CD33 chimeric receptor. The HSC are able to give rise to myeloid cells.

FIGURE 4 is a schematic showing genome editing using a CRISPR/Cas system. A sgRNA hybridizes to a portion of an exon of a lineage-specific cell-surface antigen, and the Cas9 endonuclease cleaves upstream of the Protospacer Adjacent Motif (PAM) Sequence (5'-NGG-3'). The sequences, from top to bottom, correspond to SEQ ID NOs: 45 and 46.

FIGURE 5 is a schematic showing a genome editing strategy using the CRISPR/Cas9 system to disrupt CD33. A PX458 vector encoding a Cas9 protein and a guide RNA targeting CD33 was nucleofected into K-562 cells, a human leukemic cell line. Flow cytometry was performed on the cell population using an anti-CD33 antibody prior to (top plot) and after (bottom plot) delivery of Cas9 and guide RNA to the cells. The genome editing resulted in the deletion of a coding region of the gene and a significant reduction in CD33 from the cell surface.

FIGURE 6 is a schematic showing a genome editing strategy using the CRISPR/Cas9 system to disrupt CD45RA. A PX458 vector encoding a Cas9 protein and a guide RNA targeting CD45RA was nucleofected into TIB-67 reticulum cell sarcoma mouse macrophage-like cells. Flow cytometry was performed on the cell population using an anti-CD45RA antibody prior to (top plot) and after (bottom plot) delivery of Cas9 and guide RNA to the cells. The genome editing resulted in the deletion of a coding region of the gene and a significant reduction in CD45RA from the cell surface.

FIGURE 7 shows schematics of example chimeric receptors comprising antigen-binding fragments that target CD33. A: a generic chimeric receptor targeting CD33 comprising an anti-CD33 scFv, hinge domain, transmembrane domain, co-stimulatory domain, and signaling domain. B: a chimeric receptor targeting CD33 comprising an anti-CD33 scFv, hinge domain from CD8, transmembrane domain from CD8, and intracellular domains from CD28 and CD3ζ. C: a chimeric receptor targeting CD33 comprising an anti-CD33 scFv, hinge domain from CD8, transmembrane domain from CD8, and intracellular domains from ICOS (or CD27, 4-1BB, or OX-40) and CD3ζ. D: a chimeric receptor targeting CD33 comprising an anti-CD33 scFv, hinge domain from CD8, transmembrane domain from CD8, and intracellular domains from OX40, CD28, and CD3ζ.

FIGURE 8 is a schematic of an immunotoxin.

FIGURE 9 shows expression of anti-CD33 chimeric receptors expressed in K562 cells transduced with an empty vector or vector encoding an anti-CD33 chimeric receptor. A: Western blot using a primary antibody that recognizes CD3ζ. The table provides the estimated molecular weight of each of the chimeric receptors tested. B: Flow cytometric analysis showing an increase in the population of cells that stain positive for the anti-CD33 chimeric receptor.

FIGURE 10 shows the anti-CD33 chimeric receptors bind to CD33. A: Ponceau stained protein gel. Lanes 1,3,5 : CD33 molecule. Lanes 2,4,6: CD33 mol + APC Conjugate. B: Western blot using a primary antibody that recognizes CD3ζ. Lanes 1, 3, and 5 contain the chimeric receptors co-incubated with CD33 molecules, and lanes 2, 4, and 6 contain the chimeric receptors co-incubated with a CD33-APC conjugate. C: Flow cytometric analysis showing an increase in the population of cells that express anti-CD33 chimeric receptors and bind CD33.

FIGURE 11 shows cytotoxicity of K562 cells by NK92 cells expressing the indicated chimeric receptors. A: CART1 and CART2 compared to empty HIVzsG vector. B: CART3 compared to empty HIVzsG vector.

FIGURE 12 shows cytotoxicity (expressed as percent cytotoxicity on the y-axis) of K562 cells deficient in CD33 by NK92 cells expressing the indicated chimeric receptors. A: unsorted population of K562 cells pretreated with CD33-targeting CRISPR/Cas reagents. B: single clones of K562 cells deficient in CD33. The columns, from left to right, correspond to empty HIVzsG vector, CART1, CART2, and CART3.

FIGURE 13 shows flow cytometric analysis of primary T cell populations. A: sorting of cells based on expression of T cell markers $CD4^+$, $CD8^+$, or both $CD4^+CD8^+$. B: relative expression of CD33 on the indicated populations of primary T cells.

FIGURE 14 shows cytotoxicity of K562 cells by primary T cells expressing the indicated chimeric receptors. A: $CD4^+$ T cells. B: $CD4^+/CD8^+$ (CD 4/8) and $CD8^+$ (CD8).

FIGURE 15 shows flow cytometric analysis of CD33 editing in K562 cells using the CRISPR/Cas9 system and two different gRNAs (Crispr3, right top panel, and Crispr5, right bottom panel).

FIGURE 16 shows K562 cells deficient in CD33 present normal cell proliferation and erythropoeitic differentiation. A: flow cytometric analysis of the indicated cell populations at day 1 + 50 μM hemin. B: flow cytometric analysis of the indicated cell populations at day 9. C: MTT cell proliferation assay.

FIGURE 17 shows flow cytometric analysis of CD33 editing in human $CD34^+$ cells using the CRISPR/Cas9 system and two different gRNAs (crispr3, bottom left panel, and crispr5, bottom right panel). A: flow cytometric analysis of CD33 editing in human $CD34^+$ cells using the CRISPR/Cas9 system. B: crispr3. C: crispr5.

FIGURE 18 shows colony formation for human $CD34^+/CD33^-$ cells as compared to human $CD34^+/CD33^+$ cells. The columns, from left to right, correspond to no lentivirus infection, empty vector control, crispr1, crispr 3, and crispr5.

DETAILED DESCRIPTION OF DISCLOSURE

Cancer immunotherapies targeting antigens present on the cell surface of a cancer cell is particularly challenging when the target antigen is also present on the cell surface of normal, non-cancer cells that are required or critically involved in the development and/or survival of the subject. Targeting these antigens may lead to deleterious effects in the subject due to cytotoxic effects of the immunotherapy toward such cells in addition to the cancer cells.

The methods, nucleic acids, and cells described herein allow for targeting of antigens (*e.g.*, type 1 or type 2 antigens) that are present not only on cancer cells but also cells critical for the development and/or survival of the subject. The method involves: (1) reducing the number of cells carrying the target lineage-specific cell-surface antigen using an agent that targets such an antigen; and (2) replacement of the normal cells (*e.g.*, non-cancer cells) that present the antigen and thus can be killed due to administration of the agent with hematopoietic cells that are deficient for the lineage-specific cell-surface antigen. The

methods described herein can maintain surveillance for target cells, including cancer cells, that express a lineage-specific cell-surface antigen of interest and also maintain the population of non-cancer cells expressing the lineage-specific antigen, which may be critical for development and/or survival of the subject.

Accordingly, described herein are the co-use of immune cells expressing chimeric receptors comprising an antigen-binding fragment that targets a lineage-specific cell-surface antigen such as CD33 or CD19 and hematopoietic cells such as hematopoietic stem cells (HSCs) that are deficient in the lineage-specific cell-surface antigen for treating a hematopoietic malignancy. Also provided herein are the chimeric receptors, nucleic acids encoding such, vectors comprising such, and immune cells (*e.g.*, T cells) expressing such a chimeric receptor. The present disclosure also provides genetically engineered hematopoietic cells that are deficient in a lineage-specific antigen such as those described herein, as well as methods (*e.g.*, genome editing methods) for making such.

Definitions

The terms "subject," "individual," and "patient" are used interchangeably, and refer to a vertebrate, preferably a mammal such as a human. Mammals include, but are not limited to, human primates, non-human primates or murine, bovine, equine, canine or feline species. In the context of the present disclosure, the term "subject" also encompasses tissues and cells that can be cultured in vitro or ex vivo or manipulated in vivo. The term "subject" can be used interchangeably with the term "organism".

The terms "polynucleotide", "nucleotide", "nucleotide sequence", "nucleic acid" and "oligonucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Examples of polynucleotides include, but are not limited to, coding or non-coding regions of a gene or gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. One or more nucleotides within a polynucleotide can further be modified. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may also be modified after polymerization, such as by conjugation with a labeling agent.

The term "hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the

nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogstein binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PCR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the "complement" of the given sequence.

The term "recombinant expression vector" means a genetically-modified oligonucleotide or polynucleotide construct that permits the expression of an mRNA, protein, polypeptide, or peptide by a host cell, when the construct comprises a nucleotide sequence encoding the mRNA, protein, polypeptide, or peptide, and the vector is contacted with the cell under conditions sufficient to have the mRNA, protein, polypeptide, or peptide expressed within the cell. The vectors of the present disclosure are not naturally-occurring as a whole. Parts of the vectors can be naturally-occurring. The non-naturally occurring recombinant expression vectors of the present disclosure can comprise any type of nucleotides, including, but not limited to DNA and RNA, which can be single-stranded or double-stranded, synthesized or obtained in part from natural sources, and which can contain natural, non-natural or altered nucleotides.

"Transfection," "transformation," or "transduction," as used herein, refer to the introduction of one or more exogenous polynucleotides into a host cell by using physical or chemical methods.

"Antibody," "fragment of an antibody," "antibody fragment," "functional fragment of an antibody," or "antigen-binding portion" are used interchangeably to mean one or more fragments or portions of an antibody that retain the ability to specifically bind to a specific antigen (Holliger et al., *Nat. Biotech.* (2005) 23(9): 1126). The present antibodies may be antibodies and/or fragments thereof. Antibody fragments include Fab, F(ab')2, scFv, disulfide linked Fv, Fc, or variants and/or mixtures. The antibodies may be chimeric, humanized, single chain, or bi-specific. All antibody isotypes are encompassed by the present disclosure, including, IgA, IgD, IgE, IgG, and IgM. Suitable IgG subtypes include IgG1, IgG2, IgG3 and IgG4. An antibody light or heavy chain variable region consists of a framework region interrupted by three hypervariable regions, referred to as complementarity determining regions (CDRs). The CDRs of the present antibodies or antigen-binding portions can be from a non-human or a human source. The framework of the present antibodies or antigen-binding portions can be human, humanized, non-human (e.g., a murine

framework modified to decrease antigenicity in humans), or a synthetic framework (*e.g.*, a consensus sequence).

The present antibodies or antigen-binding portions can specifically bind with a dissociation constant (K_D) of less than about 10^{-7} M, less than about 10^{-8} M, less than about 10^{-9} M, less than about 10^{-10} M, less than about 10^{-11} M, or less than about 10^{-12} M. Affinities of the antibodies according to the present disclosure can be readily determined using conventional techniques (see, *e.g.*, Scatchard et al., *Ann. N.Y. Acad. Sci.* (1949) 51:660; and U.S. Patent Nos. 5,283,173, 5,468,614, or the equivalent).

The terms "chimeric receptor," "Chimeric Antigen Receptor," or alternatively a "CAR" are used interchangeably throughout and refer to a recombinant polypeptide construct comprising at least an extracellular antigen binding domain, a transmembrane domain and a cytoplasmic signaling domain (also referred to herein as "an intracellular signaling domain") comprising a functional signaling domain derived from a stimulatory molecule as defined below. Lee et al., *Clin. Cancer Res.* (2012) 18(10):2780; Jensen et al., *Immunol Rev.* (2014) 257(1):127; www.cancer.gov/about-cancer/treatment/research/car-t-cells. In one embodiment, the stimulatory molecule is the zeta chain associated with the T cell receptor complex. In one aspect, the cytoplasmic signaling domain further comprises one or more functional signaling domains derived from at least one costimulatory molecule as defined below. The costimulatory molecule may also be 4-1BB (*i.e.*, CD137), CD27 and/or CD28 or fragments of those molecules. In another aspect, the CAR comprises a chimeric fusion protein comprising an extracellular antigen recognition domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain derived from a stimulatory molecule. The CAR comprises a chimeric fusion protein comprising an extracellular antigen recognition domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain derived from a co-stimulatory molecule and a functional signaling domain derived from a stimulatory molecule. Alternatively, the CAR comprises a chimeric fusion protein comprising an extracellular antigen recognition domain, a transmembrane domain and an intracellular signaling domain comprising two functional signaling domains derived from one or more co-stimulatory molecule(s) and a functional signaling domain derived from a stimulatory molecule. The CAR can also comprise a chimeric fusion protein comprising an extracellular antigen recognition domain, a transmembrane domain and an intracellular signaling domain comprising at least two functional signaling domains derived from one or more co-stimulatory molecule(s) and a functional signaling domain derived from a stimulatory

molecule. The antigen recognition moiety of the CAR encoded by the nucleic acid sequence can contain any lineage specific, antigen-binding antibody fragment. The antibody fragment can comprise one or more CDRs, the variable region (or portions thereof), the constant region (or portions thereof), or combinations of any of the foregoing.

The term "signaling domain" refers to the functional portion of a protein which acts by transmitting information within the cell to regulate cellular activity via defined signaling pathways by generating second messengers or functioning as effectors by responding to such messengers.

The term "zeta" or alternatively "zeta chain", "CD3-zeta" or "TCR-zeta" is defined as the protein provided as GenBank accession numbers NP_932170, NP_000725, or XP_011508447; or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like, and a "zeta stimulatory domain" or alternatively a "CD3-zeta stimulatory domain" or a "TCR-zeta stimulatory domain" is defined as the amino acid residues from the cytoplasmic domain of the zeta chain that are sufficient to functionally transmit an initial signal necessary for T cell activation.

The term "genetically engineered" or "genetically modified" refers to cells being manipulated by genetic engineering, for example by genome editing. That is, the cells contain a heterologous sequence which does not naturally occur in said cells. Typically, the heterologous sequence is introduced via a vector system or other means for introducing nucleic acid molecules into cells including liposomes. The heterologous nucleic acid molecule may be integrated into the genome of the cells or may be present extra-chromosomally, *e.g.*, in the form of plasmids. The term also includes embodiments of introducing genetically engineered, isolated CAR polypeptides into the cell.

The term "autologous" refers to any material derived from the same individual to whom it is later to be re-introduced into the same 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 be allogeneic to one another when the genes at one or more loci are not identical.

The term "cell lineage" refers to cells with a common ancestry and developing from the same type of identifiable cell into specific identifiable/functioning cells. The cell lineages used herein include, but are not limited to, respiratory, prostatic, pancreatic, mammary, renal, intestinal, neural, skeletal, vascular, hepatic, hematopoietic, muscle or cardiac cell lineages.

The term "inhibition" when used in reference to gene expression or function of a lineage specific antigen refers to a decrease in the level of gene expression or function of the

lineage specific antigen, where the inhibition is a result of interference with gene expression or function. The inhibition may be complete, in which case there is no detectable expression or function, or it may be partial. Partial inhibition can range from near complete inhibition to a near absence of inhibition. By eliminating particular target cells, CAR T cells may effectively inhibit the overall expression of particular cell lineage.

Cells such as hematopoietic cells that are "deficient in a lineage-specific antigen" refers to cells having a substantially reduced expression level of the lineage-specific antigen as compared with their naturally-occurring counterpart, *e.g.*, endogenous hematopoietic cells of the same type, or cells that do not express the lineage-specific antigen, i.e., not detectable by a routine assay such as FACS. In some instances, the express level of a lineage-specific antigen of cells that are "deficient in the antigen" can be lower than about 40% (e.g., 30%, 20%, 15%, 10%, 5% or lower) of the expression level of the same lineage-specific antigen of the naturally-occurring counterpart. As used herein, the term "about" refers to a particular value +/- 5%. For example, an expression level of about 40% may include any amount of expression between 35%-45%.

Agents targeting lineage-specific cell-surface antigens

Aspects of the disclosure provide agents targeting a lineage-specific cell-surface antigen, for example on a target cancer cell. Such an agent may comprise an antigen-binding fragment that binds and targets the lineage-specific cell-surface antigen. In some instances, the antigen-binding fragment can be a single chain antibody (scFv) specifically binding to the lineage-specific antigen.

A. Lineage-Specific Cell-Surface Antigens

As used herein, the terms "lineage-specific cell-surface antigen" and "cell-surface lineage-specific antigen" may be used interchangeably and refer to any antigen that is sufficiently present on the surface of a cell and is associated with one or more populations of cell lineage(s). For example, the antigen may be present on one or more populations of cell lineage(s) and absent (or at reduced levels) on the cell-surface of other cell populations.

In general, lineage-specific cell-surface antigens can be classified based on a number of factors such as whether the antigen and/or the populations of cells that present the antigen are required for survival and/or development of the host organism. A summary of exemplary types of lineage-specific antigens is provide in Table 1 below. See also FIGURE 1.

Table 1. Classification of Lineage Specific Antigens

Type of Lineage Specific Antigen	Characteristics of the Lineage Specific Antigen
Type 0	a) antigen is required for survival of an organism and b) cell type carrying type 0 antigen is required for survival of an organism and is not unique to a tumor, or tumor-associated virus
Type 1	a) antigen is not required for survival of an organism and b) cell type carrying type 1 antigen is not required for survival of an organism
Type 2	a) antigen is not required for survival of an organism and b) cell type carrying type 2 antigen is required for the survival of an organism
Type 3	a) antigen is not required for the survival of an organism and b) cell type carrying antigen is not required for survival of an organism c) The antigen is unique to a tumor, or a tumor associated virus An example is the LMP-2 antigen in EBV infected cells, including EBV infected tumor cells (Nasopharyngeal carcinoma and Burkitts Lymphoma)

As shown in Table 1 and FIGURE 1, type 0 lineage-specific cell-surface antigens are necessary for the tissue homeostasis and survival, and cell types carrying type 0 lineage-specific cell-surface antigen may be also necessary for survival of the subject. Thus, given the importance of type 0 lineage-specific cell-surface antigens, or cells carrying type 0 lineage-specific cell-surface antigens, in homeostasis and survival, targeting this category of antigens may be challenging using conventional CAR T cell immunotherapies, as the inhibition or removal of such antigens and cell carrying such antigens may be detrimental to the survival of the subject. Consequently, lineage-specific cell-surface antigens (such as type 0 lineage-specific antigens) and/or the cell types that carry such antigens may be required for the survival, for example because it performs a vital non-redundant function in the subject,

then this type of lineage specific antigen may be a poor target for CAR T cell based immunotherapy.

In contrast to type 0 antigens, type 1 cell-surface lineage-specific antigens and cells carrying type 1 cell-surface lineage-specific antigens are not required for tissue homeostasis or survival of the subject. Targeting type 1 cell-surface lineage-specific antigens is not likely to lead to detrimental consequences in the subject. For example, a CAR T cell engineered to target CD307, a type 1 antigen expressed uniquely on both normal plasma cells and multiple myeloma (MM) cells would lead to elimination of both cell types (FIGURE 2) (Elkins et al., *Mol Cancer Ther.* 10:2222 (2012)). However, since the plasma cell lineage is expendable for the survival of the organism, CD307 and other type 1 lineage specific antigens are antigens that are suitable for CAR T cell based immunotherapy. Lineage specific antigens of type 1 class may be expressed in a wide variety of different tissues, including, ovaries, testes, prostate, breast, endometrium, and pancreas. In some embodiments, the agent targets a cell-surface lineage-specific antigen that is a type 1 antigen.

Targeting type 2 antigens presents a significant difficulty as compared to type 1 antigens. Type 2 antigens are those characterized where: (1) the antigen is dispensable for the survival of an organism (i.e., is not required for the survival), and (2) the cell lineage carrying the antigen is indispensable for the survival of an organism (i.e., the particular cell lineage is required for the survival). For example, CD33 is a type 2 antigen expressed in both normal myeloid cells as well as in Acute Myeloid Leukemia (AML) cells (Dohner et al., *NEJM* 373:1136 (2015)). As a result, a CAR T cell engineered to target CD33 antigen could lead to the killing of both normal as well as AML cells, which may be incompatible with survival of the subject (FIGURE 3). In some embodiments, the agent targets a cell-surface lineage-specific antigen that is a type 2 antigen.

A wide variety of antigens may be targeted by the methods and compositions of the present disclosure. Monoclonal antibodies to these antigens may be purchased commercially or generated using standard techniques, including immunization of an animal with the antigen of interest followed by conventional monoclonal antibody methodologies e.g., the standard somatic cell hybridization technique of Kohler and Milstein, *Nature* (1975) 256: 495, as discussed above. The antibodies or nucleic acids encoding for the antibodies may be sequenced using any standard DNA or protein sequencing techniques.

In some embodiments, the cell-surface lineage-specific antigen that is targeted using the methods and cells described herein is a cell-surface lineage-specific antigen of leukocytes

or a subpopulation of leukocytes. In some embodiments, the cell-surface lineage-specific antigen is an antigen that is associated with myeloid cells. In some embodiments, the cell-surface lineage-specific antigen is a cluster of differentiation antigens (CDs). Examples of CD antigens include, without limitation, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3, CD3d, CD3e, CD3g, CD4, CD5, CD6, CD7, CD8a, CD8b, CD9, CD10, CD11a, CD11b, CD11c, CD11d, CDw12, CD13, CD14, CD15, CD16, CD16b, CD17, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CD32a, CD32b, CD32c, CD33, CD34, CD35, CD36, CD37, CD38, CD39, CD40, CD41, CD42a, CD42b, CD42c, CD42d, CD43, CD44, CD45, CD45RA, CD45RB, CD45RC, CD45RO, CD46, CD47, CD48, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD50, CD51, CD52, CD53, CD54, CD55, CD56, CD57, CD58, CD59, CD60a, CD61, CD62E, CD62L, CD62P, CD63, CD64a, CD65, CD65s, CD66a, CD66b, CD66c, CD66F, CD68, CD69, CD70, CD71, CD72, CD73, CD74, CD75, CD75S, CD77, CD79a, CD79b, CD80, CD81, CD82, CD83, CD84, CD85A, CD85C, CD85D, CD85E, CD85F, CD85G, CD85H, CD85I, CD85J, CD85K, CD86, CD87, CD88, CD89, CD90, CD91, CD92, CD93, CD94, CD95, CD96, CD97, CD98, CD99, CD99R, CD100, CD101, CD102, CD103, CD104, CD105, CD106, CD107a, CD107b, CD108, CD109, CD110, CD111, CD112, CD113, CD114, CD115, CD116, CD117, CD118, CD119, CD120a, CD120b, CD121a, CD121b, CD121a, CD121b, CD122, CD123, CD124, CD125, CD126, CD127, CD129, CD130, CD131, CD132, CD133, CD134, CD135, CD136, CD137, CD138, CD139, CD140a, CD140b, CD141, CD142, CD143, CD144, CDw145, CD146, CD147, CD148, CD150, CD152, CD152, CD153, CD154, CD155, CD156a, CD156b, CD156c, CD157, CD158b1, CD158b2, CD158d, CD158e1/e2, CD158f, CD158g, CD158h, CD158i, CD158j, CD158k, CD159a, CD159c, CD160, CD161, CD163, CD164, CD165, CD166, CD167a, CD168, CD169, CD170, CD171, CD172a, CD172b, CD172g, CD173, CD174, CD175, CD175s, CD176, CD177, CD178, CD179a, CD179b, CD180, CD181, CD182, CD183, CD184, CD185, CD186, CD191, CD192, CD193, CD194, CD195, CD196, CD197, CDw198, CDw199, CD200, CD201, CD202b, CD203c, CD204, CD205, CD206, CD207, CD208, CD209, CD210a, CDw210b, CD212, CD213a1, CD213a2, CD215, CD217, CD218a, CD218b, CD220, CD221, CD222, CD223, CD224, CD225, CD226, CD227, CD228, CD229, CD230, CD231, CD232, CD233, CD234, CD235a, CD235b, CD236, CD236R, CD238, CD239, CD240, CD241, CD242, CD243, CD244, CD245, CD246, CD247, CD248, CD249, CD252, CD253, CD254, CD256, CD257, CD258, CD261, CD262, CD263, CD264, CD265, CD266, CD267, CD268, CD269, CD270, CD271, CD272, CD273, CD274, CD275, CD276, CD277, CD278, CD279, CD280,

CD281, CD282, CD283, CD284, CD286, CD288, CD289, CD290, CD292, CDw293, CD294, CD295, CD296, CD297, CD298, CD299, CD300a, CD300c, CD300e, CD301, CD302, CD303, CD304, CD305, 306, CD307a, CD307b, CD307c, D307d, CD307e, CD309, CD312, CD314, CD315, CD316, CD317, CD318, CD319, CD320, CD321, CD322, CD324, CD325, CD326, CD327, CD328, CD329, CD331, CD332, CD333, CD334, CD335, CD336, CD337, CD338, CD339, CD340, CD344, CD349, CD350, CD351, CD352, CD353, CD354, CD355, CD357, CD358, CD359, CD360, CD361, CD362 and CD363. See www.bdbiosciences.com/documents/BD_Reagents_CDMarkerHuman_Poster.pdf.

In some embodiments, the cell-surface lineage-specific antigen is CD19, CD20, CD11, CD123, CD56, CD34, CD14, CD33, CD66b, CD41, CD61, CD62, CD235a, CD146, CD326, LMP2, CD22, CD52, CD10, CD3/TCR, CD79/BCR, and CD26. In some embodiments, the cell-surface lineage-specific antigen is CD33 or CD19.

Alternatively or in addition, the cell-surface lineage-specific antigen may be a cancer antigen, for example a cell-surface lineage-specific antigen that is differentially present on cancer cells. In some embodiments, the cancer antigen is an antigen that is specific to a tissue or cell lineage. Examples of cell-surface lineage-specific antigen that are associated with a specific type of cancer include, without limitation, CD20, CD22 (Non-Hodgkin's lymphoma, B-cell lymphoma, chronic lymphocytic leukemia (CLL)), CD52 (B-cell CLL), CD33 (Acute myelogenous leukemia (AML)), CD10 (gp100) (Common (pre-B) acute lymphocytic leukemia and malignant melanoma), CD3/T-cell receptor (TCR) (T-cell lymphoma and leukemia), CD79/B-cell receptor (BCR) (B-cell lymphoma and leukemia), CD26 (epithelial and lymphoid malignancies), human leukocyte antigen (HLA)-DR, HLA-DP, and HLA-DQ (lymphoid malignancies), RCAS1 (gynecological carcinomas, biliary adenocarcinomas and ductal adenocarcinomas of the pancreas) as well as prostate specific membrane antigen. In some embodiments, the cell-surface antigen CD33 and is associated with AML cells.

B. Antigen-Binding Fragment

Any antibody or an antigen-binding fragment thereof can be used for constructing the agent that targets a lineage-specific cell-surface antigen as described herein. Such an antibody or antigen-binding fragment can be prepared by a conventional method, for example, the hybridoma technology or recombinant technology.

For example, antibodies specific to a lineage-specific antigen of interest can be made by the conventional hybridoma technology. The lineage-specific antigen, which may be

coupled to a carrier protein such as KLH, can be used to immunize a host animal for generating antibodies binding to that complex. The route and schedule of immunization of the host animal are generally in keeping with established and conventional techniques for antibody stimulation and production, as further described herein. General techniques for production of mouse, humanized, and human antibodies are known in the art and are described herein. It is contemplated that any mammalian subject including humans or antibody producing cells therefrom can be manipulated to serve as the basis for production of mammalian, including human hybridoma cell lines. Typically, the host animal is inoculated intraperitoneally, intramuscularly, orally, subcutaneously, intraplantar, and/or intradermally with an amount of immunogen, including as described herein.

Hybridomas can be prepared from the lymphocytes and immortalized myeloma cells using the general somatic cell hybridization technique of Kohler, B. and Milstein, C. (1975) Nature 256:495-497 or as modified by Buck, D. W., et al., In Vitro, 18:377-381 (1982). Available myeloma lines, including but not limited to X63-Ag8.653 and those from the Salk Institute, Cell Distribution Center, San Diego, Calif., USA, may be used in the hybridization. Generally, the technique involves fusing myeloma cells and lymphoid cells using a fusogen such as polyethylene glycol, or by electrical means well known to those skilled in the art. After the fusion, the cells are separated from the fusion medium and grown in a selective growth medium, such as hypoxanthine-aminopterin-thymidine (HAT) medium, to eliminate unhybridized parent cells. Any of the media described herein, supplemented with or without serum, can be used for culturing hybridomas that secrete monoclonal antibodies. As another alternative to the cell fusion technique, EBV immortalized B cells may be used to produce the TCR-like monoclonal antibodies described herein. The hybridomas are expanded and subcloned, if desired, and supernatants are assayed for anti-immunogen activity by conventional immunoassay procedures (e.g., radioimmunoassay, enzyme immunoassay, or fluorescence immunoassay).

Hybridomas that may be used as source of antibodies encompass all derivatives, progeny cells of the parent hybridomas that produce monoclonal antibodies capable of binding to a lineage-specific antigen. Hybridomas that produce such antibodies may be grown in vitro or in vivo using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired. Undesired activity if present, can be removed, for example, by running the preparation over adsorbents made of the immunogen attached to

a solid phase and eluting or releasing the desired antibodies off the immunogen. Immunization of a host animal with a target antigen or a fragment containing the target amino acid sequence conjugated to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl, or R1N=C=NR, where R and R1 are different alkyl groups, can yield a population of antibodies (*e.g.*, monoclonal antibodies).

If desired, an antibody of interest (*e.g.*, produced by a hybridoma) may be sequenced and the polynucleotide sequence may then be cloned into a vector for expression or propagation. The sequence encoding the antibody of interest may be maintained in vector in a host cell and the host cell can then be expanded and frozen for future use. In an alternative, the polynucleotide sequence may be used for genetic manipulation to "humanize" the antibody or to improve the affinity (affinity maturation), or other characteristics of the antibody. For example, the constant region may be engineered to more resemble human constant regions to avoid immune response if the antibody is used in clinical trials and treatments in humans. It may be desirable to genetically manipulate the antibody sequence to obtain greater affinity to the lineage-specific antigen. It will be apparent to one of skill in the art that one or more polynucleotide changes can be made to the antibody and still maintain its binding specificity to the target antigen.

In other embodiments, fully human antibodies can be obtained by using commercially available mice that have been engineered to express specific human immunoglobulin proteins. Transgenic animals that are designed to produce a more desirable (*e.g.*, fully human antibodies) or more robust immune response may also be used for generation of humanized or human antibodies. Examples of such technology are XenomouseRTM from Amgen, Inc. (Fremont, Calif.) and HuMAb-MouseRTM and TC MouseTM from Medarex, Inc. (Princeton, N.J.). In another alternative, antibodies may be made recombinantly by phage display or yeast technology. See, for example, U.S. Pat. Nos. 5,565,332; 5,580,717; 5,733,743; and 6,265,150; and Winter et al., (1994) Annu. Rev. Immunol. 12:433-455, and . Alternatively, the phage display technology (McCafferty et al., (1990) Nature 348:552-553) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors.

Antigen-binding fragments of an intact antibody (full-length antibody) can be prepared via routine methods. For example, F(ab')2 fragments can be produced by pepsin digestion of an antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of F(ab')2 fragments.

Genetically engineered antibodies, such as humanized antibodies, chimeric antibodies, single-chain antibodies, and bi-specific antibodies, can be produced via, *e.g.*, conventional recombinant technology. In one example, DNA encoding a monoclonal antibodies specific to a target antigen can be readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into one or more expression vectors, which are then transfected into host cells such as E. coli cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. See, *e.g.*, PCT Publication No. WO 87/04462. The DNA can then be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, Morrison et al., (1984) *Proc. Nat. Acad. Sci.* 81:6851, or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, genetically engineered antibodies, such as "chimeric" or "hybrid" antibodies; can be prepared that have the binding specificity of a target antigen.

Techniques developed for the production of "chimeric antibodies" are well known in the art. See, *e.g.*, Morrison et al. (1984) *Proc. Natl. Acad. Sci. USA* 81, 6851; Neuberger et al. (1984) *Nature* 312, 604; and Takeda et al. (1984) *Nature* 314:452.

Methods for constructing humanized antibodies are also well known in the art. See, *e.g.*, Queen et al., *Proc. Natl. Acad. Sci. USA*, 86;10029-10033 (1989). In one example, variable regions of VH and VL of a parent non-human antibody are subjected to three-dimensional molecular modeling analysis following methods known in the art. Next, framework amino acid residues predicted to be important for the formation of the correct CDR structures are identified using the same molecular modeling analysis. In parallel, human VH and VL chains having amino acid sequences that are homologous to those of the parent non-human antibody are identified from any antibody gene database using the parent

VH and VL sequences as search queries. Human VH and VL acceptor genes are then selected.

The CDR regions within the selected human acceptor genes can be replaced with the CDR regions from the parent non-human antibody or functional variants thereof. When necessary, residues within the framework regions of the parent chain that are predicted to be important in interacting with the CDR regions (see above description) can be used to substitute for the corresponding residues in the human acceptor genes.

A single-chain antibody can be prepared via recombinant technology by linking a nucleotide sequence coding for a heavy chain variable region and a nucleotide sequence coding for a light chain variable region. Preferably, a flexible linker is incorporated between the two variable regions. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent Nos. 4,946,778 and 4,704,692) can be adapted to produce a phage or yeast scFv library and scFv clones specific to a lineage-specific antigen can be identified from the library following routine procedures. Positive clones can be subjected to further screening to identify those that bind lineage-specific antigen.

In some instances, lineage-specific antigen of interest is CD33 and the antigen-binding fragment specifically binds CD33, for example, human CD33. Amino acid and nucleic acid sequences of an exemplary heavy chain variable region and light chain variable region of an anti-human CD33 antibody are provided below. The CDR sequences are shown in boldface and underlined in the amino acid sequences.

Amino acid sequence of anti-CD33 Heavy Chain Variable Region (SEQ ID NO: 12)

QVQLQQPGAEVVKPGASVKMSCKASGYTFT**SYYIH**WIKQTPGQGLEWVG**VIYPGNDDISYNQ**
KFQGKATLTADKSSTTAYMQLSSLTSEDSAVYYCAR**EVRLRYFDV**WGQGTTVTVSS

Nucleic acid sequence of anti-CD33 Heavy Chain Variable Region (SEQ ID NO: 2)

CAGGTGCAGCTGCAGCAGCCCGGCGCCGAGGTGGTGAAGCCCGGCGCCAGCGTGAAGATGAG
CTGCAAGGCCAGCGGCTACACCTTCACCAGCTACTACATCCACTGGATCAAGCAGACCCCCG
GCCAGGGCCTGGAGTGGGTGGGCGTGATCTACCCCGGCAACGACGACATCAGCTACAACCAG
AAGTTCCAGGGCAAGGCCACCCTGACCGCCGACAAGAGCAGCACCACCGCCTACATGCAGCT
GAGCAGCCTGACCAGCGAGGACAGCGCCGTGTACTACTGCGCCAGGGAGGTGAGGCTGAGGT
ACTTCGACGTGTGGGGCCAGGGCACCACCGTGACCGTGAGCAGC

Amino acid sequence of anti-CD33 Light Chain Variable Region (SEQ ID NO: 13)

EIVLTQSPGSLAVSPGERVTMSC**KSSQSVFFSSSQKNYLA**WYQQIPGQSPRLLIY**WASTRES**
GVPDRFTGSGSGTDFTLTISSVQPEDLAIYYC**HQYLSSRT**FGQGTKLEIKR

Nucleic acid sequence of anti-CD33 Heavy Chain Variable Region (SEQ ID NO: 1)

GAGATCGTGCTGACCCAGAGCCCCGGCAGCCTGGCCGTGAGCCCCGGCGAGAGGGTGACCAT
GAGCTGCAAGAGCAGCCAGAGCGTGTTCTTCAGCAGCAGCCAGAAGAACTACCTGGCCTGGT
ACCAGCAGATCCCCGGCCAGAGCCCCAGGCTGCTGATCTACTGGGCCAGCACCAGGGAGAGC
GGCGTGCCCGACAGGTTCACCGGCAGCGGCAGCGGCACCGACTTCACCCTGACCATCAGCAG
CGTGCAGCCCGAGGACCTGGCCATCTACTACTGCCACCAGTACCTGAGCAGCAGGACCTTCG
GCCAGGGCACCAAGCTGGAGATCAAGAGG

The anti-CD33 antibody binding fragment for use in constructing the agent that targets CD33 as described herein may comprise the same heavy chain and/or light chain CDR regions as those in SEQ ID NO:12 and SEQ ID NO:13. Such antibodies may comprise amino acid residue variations in one or more of the framework regions. In some instances, the anti-CD33 antibody fragment may comprise a heavy chain variable region that shares at least 70% sequence identity (*e.g.*, 75%, 80%, 85%, 90%, 95%, or higher) with SEQ ID NO:12 and/or may comprise a light chain variable region that shares at least 70% sequence identity (*e.g.*, 75%, 80%, 85%, 90%, 95%, or higher) with SEQ ID NO:13.

The "percent identity" of two amino acid sequences is determined using the algorithm of Karlin and Altschul Proc. Natl. Acad. Sci. USA 87:2264-68, 1990, modified as in Karlin and Altschul Proc. Natl. Acad. Sci. USA 90:5873-77, 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. *J. Mol. Biol.* 215:403-10, 1990. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the protein molecules of the present disclosure. Where gaps exist between two sequences, Gapped BLAST can be utilized as described in Altschul et al., *Nucleic Acids Res.* 25(17):3389-3402, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

C. Immune Cells Expressing Chimeric Receptors

In some embodiments, the agent that targets a lineage-specific cell-surface antigen as described herein is an immune cell that expresses a chimeric receptor, which comprises an antigen-binding fragment (*e.g.*, a single-chain antibody) capable of binding to the lineage-specific antigen (*e.g.*, CD33 or CD19). Recognition of a target cell (*e.g.*, a cancer cell) having the lineage-specific antigen on its cell surface by the antigen-binding fragment of the chimeric receptor transduces an activation signal to the signaling domain(s) (*e.g.*, co-stimulatory signaling domain and/or the cytoplasmic signaling domain) of the chimeric

receptor, which may activate an effector function in the immune cell expressing the chimeric receptor.

As used herein, a chimeric receptor refers to a non-naturally occurring molecule that can be expressed on the surface of a host cell and comprises an antigen-binding fragment that binds to a cell-surface lineage-specific antigen. In general, chimeric receptors comprise at least two domains that are derived from different molecules. In addition to the antigen-binding fragment described herein, the chimeric receptor may further comprise one or more of a hinge domain, a transmembrane domain, at least one co-stimulatory domain, and a cytoplasmic signaling domain. In some embodiments, the chimeric receptor comprises from N terminus to C terminus, an antigen-binding fragment that binds to a cell-surface lineage-specific antigen, a hinge domain, a transmembrane domain, and a cytoplasmic signaling domain. In some embodiments, the chimeric receptor further comprises at least one co-stimulatory domain.

In some embodiments, the chimeric receptors described herein comprise a hinge domain, which may be located between the antigen-binding fragment and a transmembrane domain. A hinge domain is an amino acid segment that is generally found between two domains of a protein and may allow for flexibility of the protein and movement of one or both of the domains relative to one another. Any amino acid sequence that provides such flexibility and movement of the antigen-binding fragment relative to another domain of the chimeric receptor can be used.

The hinge domain may contain about 10-200 amino acids, e.g., 15-150 amino acids, 20-100 amino acids, or 30-60 amino acids. In some embodiments, the hinge domain may be of about 10, 11, 12, 13, 14,15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 amino acids in length.

In some embodiments, the hinge domain is a hinge domain of a naturally occurring protein. Hinge domains of any protein known in the art to comprise a hinge domain are compatible for use in the chimeric receptors described herein. In some embodiments, the hinge domain is at least a portion of a hinge domain of a naturally occurring protein and confers flexibility to the chimeric receptor. In some embodiments, the hinge domain is of CD8α or CD28 α. In some embodiments, the hinge domain is a portion of the hinge domain of CD8α, e.g., a fragment containing at least 15 (*e.g.*, 20, 25, 30, 35, or 40) consecutive amino acids of the hinge domain of CD8α or CD28 α.

Hinge domains of antibodies, such as an IgG, IgA, IgM, IgE, or IgD antibody, are also compatible for use in the chimeric receptors described herein. In some embodiments, the hinge domain is the hinge domain that joins the constant domains CH1 and CH2 of an antibody. In some embodiments, the hinge domain is of an antibody and comprises the hinge domain of the antibody and one or more constant regions of the antibody. In some embodiments, the hinge domain comprises the hinge domain of an antibody and the CH3 constant region of the antibody. In some embodiments, the hinge domain comprises the hinge domain of an antibody and the CH2 and CH3 constant regions of the antibody. In some embodiments, the antibody is an IgG, IgA, IgM, IgE, or IgD antibody. In some embodiments, the antibody is an IgG antibody. In some embodiments, the antibody is an IgG1, IgG2, IgG3, or IgG4 antibody. In some embodiments, the hinge region comprises the hinge region and the CH2 and CH3 constant regions of an IgG1 antibody. In some embodiments, the hinge region comprises the hinge region and the CH3 constant region of an IgG1 antibody.

Also within the scope of the present disclosure are chimeric receptors comprising a hinge domain that is a non-naturally occurring peptide. In some embodiments, the hinge domain between the C-terminus of the extracellular ligand-binding domain of an Fc receptor and the N-terminus of the transmembrane domain is a peptide linker, such as a (GlyxSer)n linker, wherein x and n, independently can be an integer between 3 and 12, including 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more.

Additional peptide linkers that may be used in a hinge domain of the chimeric receptors described herein are known in the art. See, *e.g.*, Wriggers et al. *Current Trends in Peptide Science* (2005) 80(6): 736-746 and PCT Publication WO 2012/088461.

In some embodiments, the chimeric receptors described herein may comprise a transmembrane domain. The transmembrane domain for use in the chimeric receptors can be in any form known in the art. As used herein, a "transmembrane domain" refers to any protein structure that is thermodynamically stable in a cell membrane, preferably a eukaryotic cell membrane. Transmembrane domains compatible for use in the chimeric receptors used herein may be obtained from a naturally occurring protein. Alternatively, the transmembrane domain may be a synthetic, non-naturally occurring protein segment, *e.g.*, a hydrophobic protein segment that is thermodynamically stable in a cell membrane.

Transmembrane domains are classified based on the transmembrane domain topology, including the number of passes that the transmembrane domain makes across the membrane and the orientation of the protein. For example, single-pass membrane proteins

cross the cell membrane once, and multi-pass membrane proteins cross the cell membrane at least twice (*e.g.*, 2, 3, 4, 5, 6, 7 or more times). In some embodiments, the transmembrane domain is a single-pass transmembrane domain. In some embodiments, the transmembrane domain is a single-pass transmembrane domain that orients the N terminus of the chimeric receptor to the extracellular side of the cell and the C terminus of the chimeric receptor to the intracellular side of the cell. In some embodiments, the transmembrane domain is obtained from a single pass transmembrane protein. In some embodiments, the transmembrane domain is of CD8α. In some embodiments, the transmembrane domain is of CD28. In some embodiments, the transmembrane domain is of ICOS.

In some embodiments, the chimeric receptors described herein comprise one or more costimulatory signaling domains. The term "co-stimulatory signaling domain," as used herein, refers to at least a portion of a protein that mediates signal transduction within a cell to induce an immune response, such as an effector function. The co-stimulatory signaling domain of the chimeric receptor described herein can be a cytoplasmic signaling domain from a co-stimulatory protein, which transduces a signal and modulates responses mediated by immune cells, such as T cells, NK cells, macrophages, neutrophils, or eosinophils.

In some embodiments, the chimeric receptor comprises more than one (at least 2, 3, 4, or more) co-stimulatory signaling domains. In some embodiments, the chimeric receptor comprises more than one co-stimulatory signaling domains obtained from different costimulatory proteins. In some embodiments, the chimeric receptor does not comprise a co-stimulatory signaling domain.

In general, many immune cells require co-stimulation, in addition to stimulation of an antigen-specific signal, to promote cell proliferation, differentiation and survival, and to activate effector functions of the cell. Activation of a co-stimulatory signaling domain in a host cell (*e.g.*, an immune cell) may induce the cell to increase or decrease the production and secretion of cytokines, phagocytic properties, proliferation, differentiation, survival, and/or cytotoxicity. The co-stimulatory signaling domain of any co-stimulatory protein may be compatible for use in the chimeric receptors described herein. The type(s) of co-stimulatory signaling domain is selected based on factors such as the type of the immune cells in which the chimeric receptors would be expressed (*e.g.*, primary T cells, T cell lines, NK cell lines) and the desired immune effector function (*e.g.*, cytotoxicity). Examples of co-stimulatory signaling domains for use in the chimeric receptors can be the

cytoplasmic signaling domain of co-stimulatory proteins, including, without limitation, CD27, CD28, 4-1BB, OX40, CD30, Cd40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3. In some embodiments, the co-stimulatory domain is derived from 4-1BB, CD28, or ICOS. In some embodiments, the costimulatory domain is derived from CD28 and chimeric receptor comprises a second co-stimulatory domain from 4-1BB or ICOS.

In some embodiments, the costimulatory domain is a fusion domain comprising more than one costimulatory domain or portions of more than one costimulatory domains. In some embodiments, the costimulatory domain is a fusion of costimulatory domains from CD28 and ICOS.

In some embodiments, the chimeric receptors described herein comprise a cytoplasmic signaling domain. Any cytoplasmic signaling domain can be used in the chimeric receptors described herein. In general, a cytoplasmic signaling domain relays a signal, such as interaction of an extracellular ligand-binding domain with its ligand, to stimulate a cellular response, such as inducing an effector function of the cell (e.g., cytotoxicity).

As will be evident to one of ordinary skill in the art, a factor involved in T cell activation is the phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) of a cytoplasmic signaling domain. Any ITAM-containing domain known in the art may be used to construct the chimeric receptors described herein. In general, an ITAM motif may comprise two repeats of the amino acid sequence YxxL/I separated by 6-8 amino acids, wherein each x is independently any amino acid, producing the conserved motif YxxL/Ix(6-8)YxxL/I. In some embodiments, the cytoplasmic signaling domain is from CD3ζ.

Exemplary chimeric receptors are provided in Tables 2 and 3 below.

Table 2: Exemplary components of a chimeric receptor

Chimeric receptor component	**Amino acid sequence**
Antigen-binding fragment	Light chain- GSTSSGSGKPGSGEGSTKG (SEQ ID NO: 14)-Heavy chain
CD28 costimulatory domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSP LFPGPSKPFWVLVVVGGVLACYSLLVTVA FIIFWVRSKRSRLLHSDYMNMTPRRPGPTR

	KHYQPYAPPRDFAAYRS (SEQ ID NO: 6)
ICOS costimulatory domain (boldface), ICOS transmembrane domain (italics) and a portion of the extracellular domain of ICOS (underlined)	LSIFDPPPFKVTLTGGYLHIYESQLCCQLK*F WLPIGCAAFVVVCILGCIL***CWLTKKKYSSS VHDPNGEYMFMRAVNTAKKSRLTDVTL** (SEQ ID NO: 7)
ICOS costimulatory domain	CWLTKKKYSSSVHDPNGEYMFMRAVNTA KKSRLTDVTL (SEQ ID NO: 47)
CD28/ICOS chimera (the ICOS portion shown in underline) including the hinge domain (italics) and transmembrane domain (bold) from CD28	*IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPL FPGPS***KPFWVLVVVGGVLACYSLLVTVA FIIFWV**RSKRSRLLHSDYMFMRAVNTAKK SRLTDVTL (SEQ ID NO: 8)
CD3ζ cytoplasmic signaling domain	RVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPQRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGK GHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 15)

The nucleic acid sequence of exemplary components for construction of a chimeric receptor are provided below.

CD28 intracellular signaling domain-DNA-Human (SEQ ID NO: 3)

ATTGAAGTTATGTATCCTCCTCCTTACCTAGACAATGAGAAGAGCAATGGAACCATTATCCA
TGTGAAAGGGAAACACCTTTGTCCAAGTCCCCTATTTCCCGGACCTTCTAAGCCCTTTTGGG
TGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTTATT
ATTTTCTGGGTGAGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCC
CCGCCGCCCCCGGGCCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAG
CCTATCGCTCCAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAG
AACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAG
ACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGT
ACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAG
CGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAGGACAC
CTACGACGCCCTTCACATGCAGGCCCTGCCCCCTCGC

ICOS intracellular signaling domain-DNA-Human (SEQ ID NO: 4)

CTATCAATTTTTGATCCTCCTCCTTTTAAAGTAACTCTTACAGGAGGATATTTGCATATTTA
TGAATCACAACTTTGTTGCCAGCTGAAGTTCTGGTTACCCATAGGATGTGCAGCCTTTGTTG
TAGTCTGCATTTTGGGATGCATACTTATTTGTTGGCTTACAAAAAAGAAGTATTCATCCAGT
GTGCACGACCCTAACGGTGAATACATGTTCATGAGAGCAGTGAACACAGCCAAAAAATCTAG
ACTCACAGATGTGACCCTAAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGC

AGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTG
GACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGA
AGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGA
AAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCCACC
AAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCCCCCTCGC

CD28/ICOS COSTIMULATORY SIGNALING REGION-DNA-Human (SEQ ID NO: 5)

ATTGAAGTTATGTATCCTCCTCCTTACCTAGACAATGAGAAGAGCAATGGAACCATTATCCA
TGTGAAAGGGAAACACCTTTGTCCAAGTCCCCTATTTCCCGGACCTTCTAAGCCCTTTTGGG
TGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTTATT
ATTTTCTGGGTGAGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGTTCATGAGAGC
AGTGAACACAGCCAAAAAATCTAGACTCACAGATGTGACCCTAAGAGTGAAGTTCAGCAGGA
GCGCAGACGCCCCCGCGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGA
CGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAA
GCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGG
AGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTT
TACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCC
CCCTCGC

In some embodiments, the nucleic acid sequence encodes an antigen binding fragment that binds to CD33 and comprises a heavy chain variable region which has the same CDRs as the CDRs in SEQ ID NO: 12 and a light chain variable region which has the same CDRs as the CDRs in SEQ ID NO: 13. In some embodiments, the antigen-binding fragment comprises a heavy chain variable region as provided by SEQ ID NO: 12 and a light chain variable region as provided by SEQ ID NO: 13. In some embodiments, the chimeric receptor further comprises at least a transmembrane domain and a cytoplasmic signaling domain. In some embodiments, the chimeric receptor further comprises a hinge domain and/or a co-stimulatory signaling domain.

Table 3 provides exemplary chimeric receptors described herein. The exemplary constructs have from N-terminus to C-terminus, the antigen-binding fragment, the transmembrane domain, and a cytoplasmic signaling domain. In some examples, the chimeric receptor further comprises a hinge domain located between the antigen-binding fragment and the transmembrane domain. In some example, the chimeric receptor further comprises one or more co-stimulatory domains., which may be located between the transmembrane domain and the cytoplasmic signaling domain.

Table 3: Exemplary chimeric receptors

Constructs	Vector	Antigen-binding fragment specificity	Hinge domain	Transmembrane domain	Signaling domain 1	Signaling domain 2	Signaling domain 3
CART1 (SEQ ID NO: 20)	HIVzs-Gfp	CD33	CD8α	CD8	4-1BB	CD3ζ	None
CART2 (SEQ ID NO: 21)	HIVzs-Gfp	CD33	CD8α	CD28	CD28	CD3ζ	None
CART3 (SEQ ID NO: 22)	HIVzs-Gfp	CD33	CD8α	CD28	CD28	4-1BB	CD3 ζ
CART8 (SEQ ID NO: 23)	HIVzs-Gfp	CD33	CD8α	ICOS	ICOS	4-1BB	CD3 ζ
CART4dual (SEQ ID NO: 24)	HIVzs-dT	CD19	CD8α	CD28	CD3 ζ	-	-
CART5dual (SEQ ID NO: 25)	HIVzs-Gfp	CD33	CD8α	CD28	CD28	-	-
CART6 (SEQ ID NO: 26)	HIVzs-dT	CD19	CD8α	CD28	CD28	CD3 ζ	-
CART7 (SEQ ID NO: 27)	HIVzs-Gfp	CD33	CD28hge	CD28	CD28	CD3 ζ	-

Amino acid sequences of the example chimeric receptors listed in Table 3 above are provided below:

CART1 amino acid sequence (SEQ ID NO: 20)

MWLQSLLLLGTVACSISEIVLTQSPGSLAVSPGERVTMSCKSSQSVFFSSSQKNYLAWYQQIPGQS
PRLLIYWASTRESGVPDRFTGSGSGTDFTLTISSVQPEDLAIYYCHQYLSSRTFGQGTKLEIKRGS
TSGSGKPGSGEGSTKGQVQLQQPGAEVVKPGASVKMSCKASGYTFTSYYIHWIKQTPGQGLEWVGV
IYPGNDDISYNQKFQGKATLTADKSSTTAYMQLSSLTSEDSAVYYCAREVRLRYFDVWGQGTTVTV
SSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEASRPAAGGAVHTRGLDIY
IWAPLAGTCGVLLLSLVITKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFS
RSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAY
SEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

CART2 amino acid sequence (SEQ ID NO: 21)

MWLQSLLLLGTVACSISEIVLTQSPGSLAVSPGERVTMSCKSSQSVFFSSSQKNYLAWYQQIPGQS
PRLLIYWASTRESGVPDRFTGSGSGTDFTLTISSVQPEDLAIYYCHQYLSSRTFGQGTKLEIKRGS
TSGSGKPGSGEGSTKGQVQLQQPGAEVVKPGASVKMSCKASGYTFTSYYIHWIKQTPGQGLEWVGV
IYPGNDDISYNQKFQGKATLTADKSSTTAYMQLSSLTSEDSAVYYCAREVRLRYFDVWGQGTTVTV
SALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEASRPAAGGAVHTRGLDKPF

NVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYR
SRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKD
KMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

CART3 amino acid sequence (SEQ ID NO: 22)

MWLQSLLLLGTVACSISEIVLTQSPGSLAVSPGERVTMSCKSSQSVFFSSSQKNYLAWYQQIPGQS
PRLLIYWASTRESGVPDRFTGSGSGTDFTLTISSVQPEDLAIYYCHQYLSSRTFGQGTKLEIKRGS
TSGSGKPGSGEGSTKGQVQLQQPGAEVVKPGASVKMSCKASGYTFTSYYIHWIKQTPGQGLEWVGV
IYPGNDDISYNQKFQGKATLTADKSSTTAYMQLSSLTSEDSAVYYCAREVRLRYFDVWGQGTTVTV
SSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEASRPAAGGAVHTRGLDKP
FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY
RSKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYN
ELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG
LYQGLSTATKDTYDALHMQALPPR

CART8 amino acid sequence (SEQ ID NO: 23)

MWLQSLLLLGTVACSISEIVLTQSPGSLAVSPGERVTMSCKSSQSVFFSSSQKNYLAWYQQIPGQS
PRLLIYWASTRESGVPDRFTGSGSGTDFTLTISSVQPEDLAIYYCHQYLSSRTFGQGTKLEIKRGS
TSGSGKPGSGEGSTKGQVQLQQPGAEVVKPGASVKMSCKASGYTFTSYYIHWIKQTPGQGLEWVGV
IYPGNDDISYNQKFQGKATLTADKSSTTAYMQLSSLTSEDSAVYYCAREVRLRYFDVWGQGTTVTV
SSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEASRPAAGGAVHTRGLDFW
LPIGCAAFVVVCILGCILICWLTKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTLTKRGRKKLL
YIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEY
DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK
DTYDALHMQALPPR

CART4dual amino acid sequence (SEQ ID NO: 24)

MWLQSLLLLGTVACSISIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYH
TSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEIGSTSGSGKPG
SGEGSTKGLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNS
ALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSALSNSIMY
FSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEASRPAAGGAVHTRGLDKPFWVLVVVGGV
LACYSLLVTVAFIIFWVRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP
RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

CART5dual amino acid sequence (SEQ ID NO: 25)

MWLQSLLLLGTVACSISEIVLTQSPGSLAVSPGERVTMSCRSSQSVFFSSSQKNYLAWYQQIPGQS
PRLLIYWASTRESGVPDRFTGSGSGTDFTLTISSVQPEDLAIYYCHQYLSSRTFGQGTKLEIKRGS
TSGSGKPGSGEGSTKGQVQLQQPGAEVVKPGASVKMSCKASGYTFTSYYIHWIKQTPGQGLEWVGV
IYPGNDDISYNQKFQGKATLTADKSSTTAYMQLSSLTSEDSAVYYCAREVRLRYFDVWGQGTTVTV
SSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEASRPAAGGAVHTRGLDKP
FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY
RS

CART6 amino acid sequence (SEQ ID NO: 26)

MWLQSLLLLGTVACSISIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYH
TSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEIGSTSGSGKPG
SGEGSTKGLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNS
ALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSALSNSIMY

FSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEASRPAAGGAVHTRGLDKPFWVLVVVGGV
LACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSA
DAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEI
GMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

CART7 amino acid sequence (SEQ ID NO: 27)

MWLQSLLLLGTVACSISEIVLTQSPGSLAVSPGERVTMSCKSSQSVFFSSSQKNYLAWYQQIPGQS
PRLLIYWASTRESGVPDRFTGSGSGTDFTLTISSVQPEDLAIYYCHQYLSSRTFGQGTKLEIKRGS
TSGSGKPGSGEGSTKGQVQLQQPGAEVVKPGASVKMSCKASGYTFTSYYIHWIKQTPGQGLEWVGV
IYPGNDDISYNQKFQGKATLTADKSSTTAYMQLSSLTSEDSAVYYCAREVRLRYFDVWGQGTTVTV
SSIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGVLACYSLLVTVAFIIF
WVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNE
LNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMGERRRGKGHDGLY
QGLSTATKDTYDALHMQALPPR

Nucleic acid sequences of the example chimeric receptors listed in Table 3 above are provided below:

CART1 nucleic acid sequence (SEQ ID NO: 38)

GGTGTCGTGAGCGGCCGCTGAACTGGCCACCATGTGGCTGCAGTCTCTGCTGCTGCTGGGCACCGT
GGCCTGTAGCATCAGCGAGATCGTGCTGACCCAGAGCCCTGGCTCTCTGGCTGTGTCTCCTGGCGA
GCGCGTGACCATGAGCTGCAAGAGCAGCCAGAGCGTGTTCTTCAGCAGCTCCCAGAAGAACTACCT
GGCCTGGTATCAGCAGATCCCCGGCCAGAGCCCCAGACTGCTGATCTACTGGGCCAGCACCAGAGA
AAGCGGCGTGCCCGATAGATTCACCGGCAGCGGCTCTGGCACCGACTTCACCCTGACAATCAGCAG
CGTGCAGCCCGAGGACCTGGCCATCTACTACTGCCACCAGTACCTGAGCAGCCGGACCTTTGGCCA
GGGCACCAAGCTGGAAATCAAGCGGGGCAGCACAAGCGGCAGCGGAAAGCCTGGATCTGGCGAGGG
CTCTACCAAGGGCCAGGTGCAGCTGCAGCAGCCTGGCGCCGAAGTCGTGAAACCTGGCGCCTCCGT
GAAGATGTCCTGCAAGGCCAGCGGCTACACCTTCACCAGCTACTACATCCACTGGATCAAGCAGAC
CCCTGGACAGGGCCTGGAATGGGTGGGAGTGATCTACCCCGGCAACGACGACATCAGCTACAACCA
GAAGTTCCAGGGCAAGGCCACCCTGACCGCCGACAAGTCTAGCACCACCGCCTACATGCAGCTGTC
CAGCCTGACCAGCGAGGACAGCGCCGTGTACTACTGCGCCAGAGAAGTGCGGCTGCGGTACTTCGA
TGTGTGGGGCCAGGGAACCACCGTGACCGTGTCTAGCGCCCTGAGCAACAGCATCATGTACTTCAG
CCACTTCGTGCCCGTGTTTCTGCCCGCCAAGCCTACCACAACCCCTGCCCCTAGACCTCCTACCCC
AGCCCCTACAATCGCCAGCCAGCCTCTGTCTCTGAGGCCCGAGGCTTCTAGACCAGCTGCTGGCGG
AGCCGTGCACACCAGAGGCCTGGATATCTACATCTGGGCCCCACTGGCCGGCACCTGTGGCGTGCT
GCTGCTGTCTCTCGTGATCACCAAGAGAGGCCGGAAGAAGCTGCTGTACATCTTCAAGCAGCCCTT
CATGCGGCCCGTGCAGACCACCCAGGAAGAGGACGGCTGTAGCTGCCGGTTCCCCGAGGAAGAAGA
AGGGGGCTGCGAGCTGAGAGTGAAGTTCAGCAGAAGCGCCGACGCCCCTGCCTATCAGCAGGGCCA
GAACCAGCTGTACAACGAGCTGAACCTGGGCAGACGGGAAGAGTACGACGTGCTGGACAAGCGGAG
AGGCAGGGACCCTGAGATGGGCGGCAAGCCCAGACGGAAGAACCCTCAGGAAGGCCTGTATAACGA
ACTGCAGAAAGACAAGATGGCCGAGGCCTACTCCGAGATCGGAATGAAGGGCGAGCGGAGAAGAGG
CAAGGGCCACGATGGACTGTACCAGGGCCTGAGCACCGCCACCAAGGACACCTATGACGCCCTGCA
CATGCAGGCCCTGCCCCCCAGATGAAATTCATCGACGTTAACTATTCTAG

CART2 nucleic acid sequence (SEQ ID NO: 39)

GGTGTCGTGAGCGGCCGCTGAACTGGCCACCATGTGGCTGCAGTCTCTGCTGCTGCTGGGCACCGT
GGCCTGTAGCATCAGCGAGATCGTGCTGACCCAGAGCCCTGGCTCTCTGGCTGTGTCTCCTGGCGA
GCGCGTGACCATGAGCTGCAAGAGCAGCCAGAGCGTGTTCTTCAGCAGCTCCCAGAAGAACTACCT
GGCCTGGTATCAGCAGATCCCCGGCCAGAGCCCCAGACTGCTGATCTACTGGGCCAGCACCAGAGA
AAGCGGCGTGCCCGATAGATTCACCGGCAGCGGCTCTGGCACCGACTTCACCCTGACAATCAGCAG

CGTGCAGCCCGAGGACCTGGCCATCTACTACTGCCACCAGTACCTGAGCAGCCGGACCTTTGGCCA
GGGCACCAAGCTGGAAATCAAGCGGGGCAGCACAAGCGGCAGCGGAAAGCCTGGATCTGGCGAGGG
CTCTACCAAGGGCCAGGTGCAGCTGCAGCAGCCTGGCGCCGAAGTCGTGAAACCTGGCGCCTCCGT
GAAGATGTCCTGCAAGGCCAGCGGCTACACCTTCACCAGCTACTACATCCACTGGATCAAGCAGAC
CCCTGGACAGGGCCTGGAATGGGTGGGAGTGATCTACCCCGGCAACGACGACATCAGCTACAACCA
GAAGTTCCAGGGCAAGGCCACCCTGACCGCCGACAAGTCTAGCACCACCGCCTACATGCAGCTGTC
CAGCCTGACCAGCGAGGACAGCGCCGTGTACTACTGCGCCAGAGAAGTGCGGCTGCGGTACTTCGA
TGTGTGGGGCCAGGGAACCACCGTGACCGTGTCTGCCCTGAGCAACAGCATCATGTACTTCAGCCA
CTTCGTGCCCGTGTTTCTGCCCGCCAAGCCTACCACAACCCCTGCCCCTAGACCTCCTACCCCAGC
CCCTACAATCGCCAGCCAGCCTCTGTCTCTGAGGCCCGAGGCTTCTAGACCAGCTGCTGGCGGAGC
CGTGCACACCAGAGGACTGGACAAGCCCTTCTGGGTGCTGGTGGTCGTGGGCGGAGTGCTGGCCTG
TTACAGCCTGCTCGTGACAGTGGCCTTCATCATCTTTTGGGTGCGCAGCAAGCGGTCTAGACTGCT
GCACAGCGACTACATGAACATGACCCCCAGAAGGCCAGGCCCCACCCGGAAGCACTATCAGCCTTA
CGCCCCTCCCAGAGACTTCGCCGCCTACCGGTCCAGAGTGAAGTTCAGCAGAAGCGCCGACGCCCC
TGCCTATCAGCAGGGCCAGAACCAGCTGTACAACGAGCTGAACCTGGGCAGACGGGAAGAGTACGA
CGTGCTGGACAAGAGAAGAGGCCGGGACCCTGAGATGGGCGGCAAGCCCAGACGGAAGAACCCTCA
GGAAGGCCTGTATAACGAACTGCAGAAAGACAAGATGGCCGAGGCCTACTCCGAGATCGGCATGAA
GGGCGAACGGCGGAGAGGCAAGGGACACGATGGACTGTACCAGGGCCTGAGCACCGCCACCAAGGA
CACCTATGACGCCCTGCACATGCAGGCCCTGCCCCCCAGATGAAATTCATCGACGTTAACTATTCT
AG

CART3 nucleic acid sequence (SEQ ID NO: 40)

GGTGTCGTGAGCGGCCGCTGAACTGGCCACCATGTGGCTGCAGTCTCTGCTGCTGCTGGGCACCGT
GGCCTGTAGCATCAGCGAGATCGTGCTGACCCAGAGCCCTGGCTCTCTGGCTGTGTCTCCTGGCGA
GCGCGTGACCATGAGCTGCAAGAGCAGCCAGAGCGTGTTCTTCAGCAGCTCCCAGAAGAACTACCT
GGCCTGGTATCAGCAGATCCCCGGCCAGAGCCCCAGACTGCTGATCTACTGGGCCAGCACCAGAGA
AAGCGGCGTGCCCGATAGATTCACCGGCAGCGGCTCTGGCACCGACTTCACCCTGACAATCAGCAG
CGTGCAGCCCGAGGACCTGGCCATCTACTACTGCCACCAGTACCTGAGCAGCCGGACCTTTGGCCA
GGGCACCAAGCTGGAAATCAAGCGGGGCAGCACAAGCGGCAGCGGAAAGCCTGGATCTGGCGAGGG
CTCTACCAAGGGCCAGGTGCAGCTGCAGCAGCCTGGCGCCGAAGTCGTGAAACCTGGCGCCTCCGT
GAAGATGTCCTGCAAGGCCAGCGGCTACACCTTCACCAGCTACTACATCCACTGGATCAAGCAGAC
CCCTGGACAGGGCCTGGAATGGGTGGGAGTGATCTACCCCGGCAACGACGACATCAGCTACAACCA
GAAGTTCCAGGGCAAGGCCACCCTGACCGCCGACAAGTCTAGCACCACCGCCTACATGCAGCTGTC
CAGCCTGACCAGCGAGGACAGCGCCGTGTACTACTGCGCCAGAGAAGTGCGGCTGCGGTACTTCGA
TGTGTGGGGCCAGGGAACCACCGTGACCGTGTCTAGCGCCCTGAGCAACAGCATCATGTACTTCAG
CCACTTCGTGCCCGTGTTTCTGCCCGCCAAGCCTACCACAACCCCTGCCCCTAGACCTCCTACCCC
AGCCCCTACAATCGCCAGCCAGCCTCTGTCTCTGAGGCCCGAGGCTTCTAGACCAGCTGCTGGCGG
AGCCGTGCACACCAGAGGACTGGACAAGCCCTTCTGGGTGCTGGTGGTCGTGGGCGGAGTGCTGGC
CTGTTACAGCCTGCTCGTGACAGTGGCCTTCATCATCTTTTGGGTGCGCAGCAAGCGGTCTAGACT
GCTGCACAGCGACTACATGAACATGACCCCCAGAAGGCCAGGCCCCACCCGGAAGCACTATCAGCC
TTACGCCCCTCCCAGAGACTTCGCCGCCTACAGATCCAAGAGAGGCCGGAAGAAGCTGCTGTACAT
CTTCAAGCAGCCCTTCATGCGGCCCGTGCAGACCACCCAGGAAGAGGACGGCTGTAGCTGCCGGTT
CCCCGAGGAAGAAGAAGGGGGCTGCGAGCTGAGAGTGAAGTTCAGCAGAAGCGCCGACGCCCCTGC
CTATCAGCAGGGCCAGAACCAGCTGTACAACGAGCTGAACCTGGGCAGACGGGAAGAGTACGACGT
GCTGGACAAGAGAAGAGGCCGGGACCCTGAGATGGGCGGCAAGCCCAGACGGAAGAACCCTCAGGA
AGGCCTGTATAACGAACTGCAGAAAGACAAGATGGCCGAGGCCTACTCCGAGATCGGAATGAAGGG
CGAGCGGCGGAGAGGCAAGGGACACGATGGACTGTACCAGGGCCTGAGCACCGCCACCAAGGACAC
CTATGACGCCCTGCACATGCAGGCCCTGCCCCCCAGATGAAATTCATCGACGTTAACTATTCTAG

CART4dual nucleic acid sequence (SEQ ID NO: 41)

GGTGTCGTGAGCGGCCGCTGAACTGGCCACCATGTGGCTGCAGTCTCTGCTGCTGCTGGGCACCGT
GGCCTGCAGCATCAGCATCCAGATGACCCAGACCACCAGCAGCCTGAGCGCCAGCCTGGGCGATAG
AGTGACCATCAGCTGCAGAGCCAGCCAGGACATCAGCAAGTACCTGAACTGGTATCAGCAGAAACC

CGACGGCACCGTGAAGCTGCTGATCTACCACACCAGCAGACTGCACAGCGGCGTGCCCTCTAGATT
TTCCGGCAGCGGCTCCGGCACCGACTACAGCCTGACCATCTCCAACCTGGAACAGGAAGATATCGC
TACCTACTTCTGTCAGCAAGGCAACACCCTGCCCTACACCTTCGGCGGAGGCACCAAGCTGGAAAT
CGGCAGCACAAGCGGCTCTGGCAAGCCTGGATCTGGCGAGGGCTCTACCAAGGGCCTGCAGGAATC
TGGCCCTGGACTGGTGGCCCCTAGCCAGAGCCTGTCTGTGACCTGTACCGTGTCCGGCGTGTCCCT
GCCTGACTATGGCGTGTCCTGGATCAGACAGCCCCCCAGAAAGGGCCTGGAATGGCTGGGAGTGAT
CTGGGGCAGCGAGACAACCTACTACAACAGCGCCCTGAAGTCCCGGCTGACCATCATCAAGGACAA
CTCCAAGAGCCAGGTGTTCCTGAAGATGAACAGCCTGCAGACCGACGACACCGCCATCTACTACTG
CGCCAAGCACTACTACTACGGCGGCAGCTACGCCATGGACTACTGGGGCCAGGGCACAAGCGTGAC
CGTGTCTGCCCTGAGCAACAGCATCATGTACTTCAGCCACTTCGTGCCCGTGTTTCTGCCCGCCAA
GCCTACCACAACCCCTGCCCCTAGACCTCCTACCCCAGCCCCTACAATCGCCAGCCAGCCTCTGTC
TCTGAGGCCCGAGGCTTCTAGACCAGCTGCTGGCGGAGCCGTGCACACCAGAGGACTGGACAAGCC
CTTCTGGGTGCTGGTGGTCGTGGGCGGAGTGCTGGCCTGTTATAGCCTGCTCGTGACAGTGGCCTT
CATCATCTTTTGGGTGCGCGTGAAGTTCAGCCGCAGCGCCGATGCCCCTGCCTATCAGCAGGGACA
GAACCAGCTGTACAACGAGCTGAACCTGGGCAGACGGGAAGAGTACGACGTGCTGGACAAGAGAAG
AGGCCGGGACCCTGAGATGGGCGGCAAGCCCAGAAGAAAGAACCCCCAGGAAGGCCTGTATAACGA
ACTGCAGAAAGACAAGATGGCCGAGGCCTACAGCGAGATCGGCATGAAGGGCGAACGGCGGAGAGG
CAAGGGCCACGATGGACTGTATCAGGGCCTGAGCACCGCCACCAAGGACACCTATGACGCCCTGCA
CATGCAGGCTCTGCCCCCTCGCTGAAATTCATCGACGTTAACTATTCTAG

CART5dual nucleic acid sequence (SEQ ID NO: 42)

GGTGTCGTGAGCGGCCGCTGAACTGGCCACCATGTGGCTGCAGTCTCTGCTGCTGCTGGGCACCGT
GGCCTGTAGCATCAGCGAGATCGTGCTGACCCAGAGCCCTGGCTCTCTGGCTGTGTCTCCTGGCGA
GCGCGTGACCATGAGCTGCAAGAGCAGCCAGAGCGTGTTCTTCAGCAGCTCCCAGAAGAACTACCT
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AAGCGGCGTGCCCGATAGATTCACCGGCAGCGGCTCTGGCACCGACTTCACCCTGACAATCAGCAG
CGTGCAGCCCGAGGACCTGGCCATCTACTACTGCCACCAGTACCTGAGCAGCCGGACCTTTGGCCA
GGGCACCAAGCTGGAAATCAAGCGGGGCAGCACAAGCGGCAGCGGAAAGCCTGGATCTGGCGAGGG
CTCTACCAAGGGCCAGGTGCAGCTGCAGCAGCCTGGCGCCGAAGTCGTGAAACCTGGCGCCTCCGT
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CCCTGGACAGGGCCTGGAATGGGTGGGAGTGATCTACCCCGGCAACGACGACATCAGCTACAACCA
GAAGTTCCAGGGCAAGGCCACCCTGACCGCCGACAAGTCTAGCACCACCGCCTACATGCAGCTGTC
CAGCCTGACCAGCGAGGACAGCGCCGTGTACTACTGCGCCAGAGAAGTGCGGCTGCGGTACTTCGA
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CCACTTCGTGCCCGTGTTTCTGCCCGCCAAGCCTACCACAACCCCTGCCCCTAGACCTCCTACCCC
AGCCCCTACAATCGCCAGCCAGCCTCTGTCTCTGAGGCCCGAGGCTTCTAGACCAGCTGCTGGCGG
AGCCGTGCACACCAGAGGACTGGACAAGCCCTTCTGGGTGCTGGTGGTCGTGGGCGGAGTGCTGGC
CTGTTACAGCCTGCTCGTGACAGTGGCCTTCATCATCTTTTGGGTGCGCAGCAAGCGGTCTAGACT
GCTGCACAGCGACTACATGAACATGACCCCCAGAAGGCCAGGCCCCACCCGGAAGCACTATCAGCC
TTACGCCCCTCCCAGAGACTTCGCCGCCTACAGAAGCTGAAATTCATCGACGTTAACTATTCTAG

CART6 nucleic acid sequence (SEQ ID NO: 43)

GGTGTCGTGAGCGGCCGCTGAACTGGCCACCATGTGGCTGCAGTCTCTGCTGCTGCTGGGCACCGT
GGCCTGCAGCATCAGCATCCAGATGACCCAGACCACCAGCAGCCTGAGCGCCAGCCTGGGCGATAG
AGTGACCATCAGCTGCAGAGCCAGCCAGGACATCAGCAAGTACCTGAACTGGTATCAGCAGAAACC
CGACGGCACCGTGAAGCTGCTGATCTACCACACCAGCAGACTGCACAGCGGCGTGCCCTCTAGATT
TTCCGGCAGCGGCTCCGGCACCGACTACAGCCTGACCATCTCCAACCTGGAACAGGAAGATATCGC
TACCTACTTCTGTCAGCAAGGCAACACCCTGCCCTACACCTTCGGCGGAGGCACCAAGCTGGAAAT
CGGCAGCACAAGCGGCTCTGGCAAGCCTGGATCTGGCGAGGGCTCTACCAAGGGCCTGCAGGAATC
TGGCCCTGGACTGGTGGCCCCTAGCCAGAGCCTGTCTGTGACCTGTACCGTGTCCGGCGTGTCCCT
GCCTGACTATGGCGTGTCCTGGATCAGACAGCCCCCCAGAAAGGGCCTGGAATGGCTGGGAGTGAT
CTGGGGCAGCGAGACAACCTACTACAACAGCGCCCTGAAGTCCCGGCTGACCATCATCAAGGACAA
CTCCAAGAGCCAGGTGTTCCTGAAGATGAACAGCCTGCAGACCGACGACACCGCCATCTACTACTG

CGCCAAGCACTACTACTACGGCGGCAGCTACGCCATGGACTACTGGGGCCAGGGCACAAGCGTGAC
CGTGTCTGCCCTGAGCAACAGCATCATGTACTTCAGCCACTTCGTGCCCGTGTTTCTGCCCGCCAA
GCCTACCACAACCCCTGCCCCTAGACCTCCTACCCCAGCCCCTACAATCGCCAGCCAGCCTCTGTC
TCTGAGGCCCGAGGCTTCTAGACCAGCTGCTGGCGGAGCCGTGCACACCAGAGGACTGGACAAGCC
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CATCATCTTTTGGGTGCGCAGCAAGCGGAGCCGGCTGCTGCACTCCGACTACATGAACATGACCCC
CAGACGGCCAGGCCCCACCCGGAAACACTATCAGCCTTACGCCCCTCCCAGAGACTTCGCCGCCTA
CCGGTCCAGAGTGAAGTTCAGCAGATCCGCCGACGCCCCTGCCTATCAGCAGGGACAGAACCAGCT
GTACAACGAGCTGAACCTGGGCAGACGGGAAGAGTACGACGTGCTGGACAAGAGAAGAGGCCGGGA
CCCTGAGATGGGCGGCAAGCCCAGAAGAAAGAACCCCCAGGAAGGCCTGTATAACGAACTGCAGAA
AGACAAGATGGCCGAGGCCTACAGCGAGATCGGCATGAAGGGCGAACGGCGGAGAGGCAAGGGCCA
CGATGGACTGTATCAGGGCCTGAGCACCGCCACCAAGGACACCTATGACGCCCTGCACATGCAGGC
TCTGCCCCCTCGCTGAAATTCATCGACGTTAACTATTCTAG

CART7 nucleic acid sequence (SEQ ID NO: 44)

GGTGTCGTGAGCGGCCGCTGAACTGGCCACCATGTGGCTGCAGTCTCTGCTGCTGCTGGGCACCGT
GGCCTGTAGCATCAGCGAGATCGTGCTGACCCAGAGCCCTGGCTCTCTGGCTGTGTCTCCTGGCGA
GCGCGTGACCATGAGCTGCAAGAGCAGCCAGAGCGTGTTCTTCAGCAGCTCCCAGAAGAACTACCT
GGCCTGGTATCAGCAGATCCCCGGCCAGAGCCCCAGACTGCTGATCTACTGGGCCAGCACCAGAGA
AAGCGGCGTGCCCGATAGATTCACCGGCAGCGGCTCTGGCACCGACTTCACCCTGACAATCAGCAG
CGTGCAGCCCGAGGACCTGGCCATCTACTACTGCCACCAGTACCTGAGCAGCCGGACCTTTGGCCA
GGGCACCAAGCTGGAAATCAAGCGGGGCAGCACAAGCGGCAGCGGAAAGCCTGGATCTGGCGAGGG
CTCTACCAAGGGCCAGGTGCAGCTGCAGCAGCCTGGCGCCGAAGTCGTGAAACCTGGCGCCTCCGT
GAAGATGTCCTGCAAGGCCAGCGGCTACACCTTCACCAGCTACTACATCCACTGGATCAAGCAGAC
CCCTGGACAGGGCCTGGAATGGGTGGGAGTGATCTACCCCGGCAACGACGACATCAGCTACAACCA
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CAGCCTGACCAGCGAGGACAGCGCCGTGTACTACTGCGCCAGAGAAGTGCGGCTGCGGTACTTCGA
TGTGTGGGGCCAGGGAACCACCGTGACCGTGTCCAGCATCGAAGTGATGTACCCGCCTCCCTACCT
GGACAACGAGAAGTCCAACGGCACCATCATCCACGTGAAGGGCAAGCACCTGTGCCCCAGCCCTCT
GTTTCCTGGCCCTAGCAAGCCCTTCTGGGTGCTGGTGGTCGTGGGCGGAGTGCTGGCCTGTTACAG
CCTGCTCGTGACAGTGGCCTTCATCATCTTTTGGGTGCGCAGCAAGCGGTCTAGACTGCTGCACAG
CGACTACATGAACATGACCCCCAGAAGGCCAGGCCCCACCCGGAAGCACTATCAGCCTTACGCCCC
TCCCAGAGACTTCGCCGCCTACCGGTCCAGAGTGAAGTTCAGCAGAAGCGCCGACGCCCCTGCCTA
TCAGCAGGGCCAGAACCAGCTGTACAACGAGCTGAACCTGGGCAGACGGGAAGAGTACGACGTGCT
GGACAAGCGGAGAGGCAGGGACCCTGAGATGGGCGGCAAGCCCAGACGGAAGAACCCTCAGGAAGG
CCTGTATAACGAACTGCAGAAAGACAAGATGGCCGAGGCCTACTCCGAGATCGGCATGAAGGGCGA
GCGGAGAAGAGGCAAGGGCCACGATGGACTGTACCAGGGCCTGAGCACCGCCACCAAGGACACCTA
TGACGCCCTGCACATGCAGGCCCTGCCCCCCAGATGAAATTCATCGACGTTAACTATTCTAG

Any of the chimeric receptors described herein can be prepared by routine methods, such as recombinant technology. Methods for preparing the chimeric receptors herein involve generation of a nucleic acid that encodes a polypeptide comprising each of the domains of the chimeric receptors, including the antigen-binding fragment and optionally, the hinge domain, the transmembrane domain, at least one co-stimulatory signaling domain, and the cytoplasmic signaling domain. In some embodiments, a nucleic acid encoding each of the components of chimeric receptor are joined together using recombinant technology.

Sequences of each of the components of the chimeric receptors may be obtained via routine technology, *e.g.*, PCR amplification from any one of a variety of sources known in the art. In some embodiments, sequences of one or more of the components of the chimeric receptors are obtained from a human cell. Alternatively, the sequences of one or more components of the chimeric receptors can be synthesized. Sequences of each of the components (*e.g.*, domains) can be joined directly or indirectly (*e.g.*, using a nucleic acid sequence encoding a peptide linker) to form a nucleic acid sequence encoding the chimeric receptor, using methods such as PCR amplification or ligation. Alternatively, the nucleic acid encoding the chimeric receptor may be synthesized. In some embodiments, the nucleic acid is DNA. In other embodiments, the nucleic acid is RNA.

Mutation of one or more residues within one or more of the components of the chimeric receptor (*e.g.*, the antigen-binding fragment, etc), prior to or after joining the sequences of each of the components. In some embodiments, one or more mutations in a component of the chimeric receptor may be made to modulate (increase or decrease) the affinity of the component for a target (*e.g.*, the antigen-binding fragment for the target antigen) and/or modulate the activity of the component.

Any of the chimeric receptors described herein can be introduced into a suitable immune cell for expression via conventional technology. In some embodiments, the immune cells are T cells, such as primary T cells or T cell lines. Alternatively, the immune cells can be NK cells, such as established NK cell lines (*e.g.*, NK-92 cells). In some embodiments, the immune cells are T cells that express CD8 ($CD8^+$) or CD8 and CD4 ($CD8^+/CD4^+$). In some embodiments, the T cells are T cells of an established T cell line, for example, 293T cells or Jurkat cells.

Primary T cells may be obtained from any source, such as peripheral blood mononuclear cells (PBMCs), bone marrow, tissues such as spleen, lymph node, thymus, or tumor tissue. A source suitable for obtaining the type of immune cells desired would be evident to one of skill in the art. In some embodiments, the population of immune cells is derived from a human patient having a hematopoietic malignancy, such as from the bone marrow or from PBMCs obtained from the patient. In some embodiments, the population of immune cells is derived from a healthy donor. In some embodiments, the immune cells are obtained from the subject to whom the immune cells expressing the chimeric receptors will be subsequently administered. Immune cells that are administered to the same subject from which the cells were obtained are referred to as autologous cells, whereas immune cells that

are obtained from a subject who is not the subject to whom the cells will be administered are referred to as allogeneic cells.

The type of host cells desired may be expanded within the population of cells obtained by co-incubating the cells with stimulatory molecules, for example, anti-CD3 and anti-CD28 antibodies may be used for expansion of T cells.

To construct the immune cells that express any of the chimeric receptor constructs described herein, expression vectors for stable or transient expression of the chimeric receptor construct may be constructed via conventional methods as described herein and introduced into immune host cells. For example, nucleic acids encoding the chimeric receptors may be cloned into a suitable expression vector, such as a viral vector in operable linkage to a suitable promoter. The nucleic acids and the vector may be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of the nucleic acid encoding the chimeric receptors. The synthetic linkers may contain nucleic acid sequences that correspond to a particular restriction site in the vector. The selection of expression vectors/plasmids/viral vectors would depend on the type of host cells for expression of the chimeric receptors, but should be suitable for integration and replication in eukaryotic cells.

A variety of promoters can be used for expression of the chimeric receptors described herein, including, without limitation, cytomegalovirus (CMV) intermediate early promoter, a viral LTR such as the Rous sarcoma virus LTR, HIV-LTR, HTLV-1 LTR, Maloney murine leukemia virus (MMLV) LTR, myeoloproliferative sarcoma virus (MPSV) LTR, spleen focus-forming virus (SFFV) LTR, the simian virus 40 (SV40) early promoter, herpes simplex tk virus promoter, elongation factor 1-alpha (EF1-α) promoter with or without the EF1-α intron. Additional promoters for expression of the chimeric receptors include any constitutively active promoter in an immune cell. Alternatively, any regulatable promoter may be used, such that its expression can be modulated within an immune cell.

Additionally, the vector may contain, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in host cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; 5'-and 3'-untranslated regions for mRNA stability and translation efficiency from highly-expressed genes like α-globin or β-globin; SV40 polyoma origins of replication and ColE1 for proper episomal replication; internal ribosome

binding sites (IRESes), versatile multiple cloning sites; T7 and SP6 RNA promoters for in vitro transcription of sense and antisense RNA; a "suicide switch" or "suicide gene" which when triggered causes cells carrying the vector to die (e.g., HSV thymidine kinase, an inducible caspase such as iCasp9), and reporter gene for assessing expression of the chimeric receptor. See section VI below. Suitable vectors and methods for producing vectors containing transgenes are well known and available in the art. Examples of the preparation of vectors for expression of chimeric receptors can be found, for example, in US2014/0106449, herein incorporated by reference in its entirety.

In some embodiments, the chimeric receptor construct or the nucleic acid encoding said chimeric receptor is a DNA molecule. In some embodiments, chimeric receptor construct or the nucleic acid encoding said chimeric receptor is a DNA vector and may be electroporated to immune cells (see, e.g., Till, et al. Blood (2012) 119(17): 3940-3950). In some embodiments, the nucleic acid encoding the chimeric receptor is an RNA molecule, which may be electroporated to immune cells.

Any of the vectors comprising a nucleic acid sequence that encodes a chimeric receptor construct described herein is also within the scope of the present disclosure. Such a vector may be delivered into host cells such as host immune cells by a suitable method. Methods of delivering vectors to immune cells are well known in the art and may include DNA, RNA, or transposon electroporation, transfection reagents such as liposomes or nanoparticles to delivery DNA, RNA, or transposons; delivery of DNA, RNA, or transposons or protein by mechanical deformation (see, *e.g.*, Sharei et al. *Proc. Natl. Acad. Sci. USA* (2013) 110(6): 2082-2087); or viral transduction. In some embodiments, the vectors for expression of the chimeric receptors are delivered to host cells by viral transduction. Exemplary viral methods for delivery include, but are not limited to, recombinant retroviruses (see, *e.g.*, PCT Publication Nos. WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; WO 93/11230; WO 93/10218; WO 91/02805; U.S. Pat. Nos. 5,219,740 and 4,777,127; GB Patent No. 2,200,651; and EP Patent No. 0 345 242), alphavirus-based vectors, and adeno-associated virus (AAV) vectors (see, *e.g.*, PCT Publication Nos. WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655). In some embodiments, the vectors for expression of the chimeric receptors are retroviruses. In some embodiments, the vectors for expression of the chimeric receptors are lentiviruses. In some embodiments, the vectors for expression of the chimeric receptors are adeno-associated viruses.

In examples in which the vectors encoding chimeric receptors are introduced to the host cells using a viral vector, viral particles that are capable of infecting the immune cells and carry the vector may be produced by any method known in the art and can be found, for example in PCT Application No. WO 1991/002805A2, WO 1998/009271 A1, and U.S. Patent 6,194,191. The viral particles are harvested from the cell culture supernatant and may be isolated and/or purified prior to contacting the viral particles with the immune cells.

The methods of preparing host cells expressing any of the chimeric receptors described herein may comprise activating and/or expanding the immune cells *ex vivo*. Activating a host cell means stimulating a host cell into an activate state in which the cell may be able to perform effector functions (*e.g.*, cytotoxicity). Methods of activating a host cell will depend on the type of host cell used for expression of the chimeric receptors. Expanding host cells may involve any method that results in an increase in the number of cells expressing chimeric receptors, for example, allowing the host cells to proliferate or stimulating the host cells to proliferate. Methods for stimulating expansion of host cells will depend on the type of host cell used for expression of the chimeric receptors and will be evident to one of skill in the art. In some embodiments, the host cells expressing any of the chimeric receptors described herein are activated and/or expanded *ex vivo* prior to administration to a subject.

In some embodiments, the agents targeting a cell-surface lineage-specific antigen is an antibody-drug conjugate (ADC). As will be evident to one of ordinary skill in the art, the term "antibody-drug conjugate" can be used interchangeably with "immunotoxin" and refers to a fusion molecule comprising an antibody (or antigen-binding fragment thereof) conjugated to a toxin or drug molecule. Binding of the antibody to the corresponding antigen allows for delivery of the toxin or drug molecule to a cell that presents the antigen on the its cell surface (*e.g.*, target cell), thereby resulting in death of the target cell.

In some embodiments, the agent is an antibody-drug conjugate. In some embodiments, the antibody-drug conjugate comprises an antigen-binding fragment and a toxin or drug that induces cytotoxicity in a target cell. In some embodiments, the antibody-drug conjugate targets a type 2 antigen. In some embodiments, the antibody-drug conjugate targets CD33 or CD19.

In some embodiments, the antigen-bind fragment of the antibody-drug conjugate has the same heavy chain CDRs as the heavy chain variable region provided by SEQ ID NO: 12 and the same light chain CDRS as the light chain variable region provided by SEQ ID NO: 13. In some embodiments, the antigen-bind fragment of the antibody-drug conjugate has the

heavy chain variable region provided by SEQ ID NO: 12 and the same light chain variable region provided by SEQ ID NO: 13.

Toxins or drugs compatible for use in antibody-drug conjugate are well known in the art and will be evident to one of ordinary skill in the art. See, *e.g.*, Peters et al. *Biosci. Rep.*(2015) 35(4): e00225. In some embodiments, the antibody-drug conjugate may further comprise a linker (*e.g.*, a peptide linker, such as a cleavable linker) attaching the antibody and drug molecule.

An ADC described herein may be used as a follow-on treatment to subjects who have been undergone the combined therapy as described herein.

Hematopoietic Cells Deficient in a Lineage-Specific Cell-Surface Antigen

The present disclosure also provides hematopoietic cells such as HSCs that have been genetically modified to be deficient in a lineage-specific cell-surface antigen. In some embodiments, the hematopoietic cells are hematopoietic stem cells. Hematopoietic stem cells (HSCs) are capable of giving rise to both myeloid and lymphoid progenitor cells that further give rise to myeloid cells (*e.g.*, monocytes, macrophages, neutrophils, basophils, dendritic cells, erythrocytes, platelets, etc) and lymphoid cells (*e.g.*, T cells, B cells, NK cells), respectively. HSCs are characterized by the expression of the cell surface marker CD34 (*e.g.*, $CD34^+$), which can be used for the identification and/or isolation of HSCs, and absence of cell surface markers associated with commitment to a cell lineage.

In some embodiments, the HSCs are obtained from a subject, such as a mammalian subject. In some embodiments, the mammalian subject is a non-human primate, a rodent (*e.g.*, mouse or rat), a bovine, a porcine, an equine, or a domestic animal. In some embodiments, the HSCs are obtained from a human patient, such as a human patient having a hematopoietic malignancy. In some embodiments, the HSCs are obtained from a healthy donor. In some embodiments, the HSCs are obtained from the subject to whom the immune cells expressing the chimeric receptors will be subsequently administered. HSCs that are administered to the same subject from which the cells were obtained are referred to as autologous cells, whereas HSCs that are obtained from a subject who is not the subject to whom the cells will be administered are referred to as allogeneic cells.

HSCs may be obtained from any suitable source using convention means known in the art. In some embodiments, HSCs are obtained from a sample from a subject, such as bone marrow sample or from a blood sample. Alternatively or in addition, HSCs may be obtained from an umbilical cord. In some embodiments, the HSCs are from bone marrow or

peripheral blood mononuclear cells (PBMCs). In general, bone marrow cells may be obtained from iliac crest, femora, tibiae, spine, rib or other medullary spaces of a subject. Bone marrow may be taken out of the patient and isolated through various separations and washing procedures known in the art. An exemplary procedure for isolation of bone marrow cells comprises the following steps: a) extraction of a bone marrow sample; b) centrifugal separation of bone marrow suspension in three fractions and collecting the intermediate fraction, or buffycoat; c) the buffycoat fraction from step (b) is centrifuged one more time in a separation fluid, commonly Ficoll(TM), and an intermediate fraction which contains the bone marrow cells is collected; and d) washing of the collected fraction from step (c) for recovery of re-transfusable bone marrow cells.

HSCs typically reside in the bone marrow but can be mobilized into the circulating blood by administering a mobilizing agent in order to harvest HSCs from the peripheral blood. In some embodiments, the subject from which the HSCs are obtained is administered a mobilizing agent, such as granulocyte colony-stimulating factor (G-CSF). The number of the HSCs collected following mobilization using a mobilizing agent is typically greater than the number of cells obtained without use of a mobilizing agent.

In some embodiments, a sample is obtained from a subject and is then enriched for a desired cell type (*e.g.* $CD34^+/CD33^-$ cells). For example, PBMCs and/or $CD34^+$ hematopoietic cells can be isolated from blood as described herein. Cells can also be isolated from other cells, for example by isolation and/or activation with an antibody binding to an epitope on the cell surface of the desired cell type. Another method that can be used includes negative selection using antibodies to cell surface markers to selectively enrich for a specific cell type without activating the cell by receptor engagement.

Populations of HSC can be expanded prior to or after genetically engineering the HSC to become deficient in a lineage specific cell-surface antigen. The cells may be cultured under conditions that comprise an expansion medium comprising one or more cytokines, such as stem cell factor (SCF), Flt-3 ligand (Flt3L), thrombopoietin (TPO), Interleukin 3 (IL-3), or Interleukin 6 (IL-6). The cell may be expanded for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 days or any range necessary. In some embodiments, the HSC are expanded after isolation of a desired cell population (*e.g.*, $CD34^+/CD33^-$) from a sample obtained from a subject and prior to genetic engineering. In some embodiments, the HSC are expanded after genetic engineering, thereby selectively expanding cells that have undergone the genetic modification and are deficient in a lineage-specific cell-surface antigen. In some embodiments, a cell ("a clone") or several cells having

a desired characteristic (*e.g.*, phenotype or genotype) following genetic modification may be selected and independently expanded.

In some embodiments, the hematopoietic cells are genetically engineered to be deficient in a cell-surface lineage-specific antigen. In some embodiments, the hematopoietic cells are genetically engineered to be deficient in the same cell-surface lineage-specific antigen that is targeted by the agent. As used herein, a hematopoietic cell is considered to be deficient in a cell-surface lineage-specific antigen if hematopoietic cell has substantially reduced expression of the cell-surface lineage-specific antigen as compared to a naturally-occurring hematopoietic cell of the same type as the genetically engineered hematopoietic cell (*e.g.*, is characterized by the presence of the same cell surface markers, such as CD34). In some embodiments, the hematopoietic cell has no detectable expression of the cell-surface lineage-specific antigen. The expression level of a cell-surface lineage-specific antigen can be assessed by any means known in the art. For example, the expression level of a cell-surface lineage-specific antigen can be assessed by detecting the antigen with an antigen-specific antibody (*e.g.*, flow cytometry methods, Western blotting).

In some embodiments, the expression of the cell-surface lineage-specific antigen on the genetically engineered hematopoietic cell is compared to the expression of the cell-surface lineage-specific antigen on a naturally occurring hematopoietic cell. In some embodiments, the genetic engineering results in a reduction in the expression level of the cell-surface lineage-specific antigen by at least about 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% as compared to the expression of the cell-surface lineage-specific antigen on a naturally occurring hematopoietic cell.

In some embodiments, the hematopoietic cell is deficient in the whole endogenous gene encoding the cell-surface lineage-specific antigen. In some embodiments, the whole endogenous gene encoding the cell-surface lineage-specific antigen has been deleted. In some embodiments, the hematopoietic cell comprises a portion of endogenous gene encoding the cell-surface lineage-specific antigen. In some embodiments, the hematopoietic cell expressing a portion (*e.g.* a truncated protein) of the cell-surface lineage-specific antigen. In other embodiments, a portion of the endogenous gene encoding the cell-surface lineage-specific antigen has been deleted. In some embodiments, at least 10%, 20%, 30%, 40%, 50%, 60%, 70% or more of the gene encoding the cell-surface lineage-specific antigen has been deleted.

As will be appreciated by one of ordinary skill in the art, a portion of the nucleotide sequence encoding the cell-surface lineage-specific antigen may be deleted or one or more

non-coding sequences, such that the hematopoietic cell is deficient in the antigen (e.g., has substantially reduced expression of the antigen).

In some embodiments, the cell-surface lineage-specific antigen is CD33. The predicted structure of CD33 includes two immunoglobulin domains, an IgV domain and an IgC2 domain. In some embodiments, a portion of the immunoglobulin C domain of CD33 is deleted.

Any of the genetically engineering hematopoietic cells, such as HSCs, that are deficient in a cell-surface lineage-specific antigen can be prepared by a routine method or by a method described herein. In some embodiments, the genetic engineering is performed using genome editing. As used herein, "genome editing" refers to a method of modifying the genome, including any protein-coding or non-coding nucleotide sequence, of an organism to knock out the expression of a target gene. In general, genome editing methods involve use of an endonuclease that is capable of cleaving the nucleic acid of the genome, for example at a targeted nucleotide sequence. Repair of the double-stranded breaks in the genome may be repaired introducing mutations and/or exogenous nucleic acid may be inserted into the targeted site.

Genome editing methods are generally classified based on the type of endonuclease that is involved in generating double stranded breaks in the target nucleic acid. These methods include use of zinc finger nucleases (ZFN), transcription activator-like effector-based nuclease (TALEN), meganucleases, and CRISPR/Cas systems.

In one aspect of the present disclosure, the replacement of the tumor cells by a modified population of normal cells is performed using normal cells in which a lineage-specific antigen is modified. Such modification may include the depletion or inhibition of any lineage specific antigen using a CRISPR-Cas9 system, where the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 system is an engineered, non-naturally occurring CRISPR-Cas9 system (FIGURE 4).

CRISPR-Cas system has been successfully utilized to edit the genomes of various organisms, including, but not limited to bacteria, humans, fruit flies, zebra fish and plants. See, *e.g.*, Jiang et al., *Nature Biotechnology* (2013) 31(3):233; Qi et al, *Cell* (2013) 5:1173; DiCarlo et al., *Nucleic Acids Res.* (2013) 7:4336; Hwang et al., *Nat. Biotechnol* (2013), 3:227); Gratz et al., *Genetics* (2013) 194:1029; Cong et al., *Science* (2013) 6121:819; Mali et al., *Science* (2013) 6121:823; Cho et al. *Nat. Biotechnol* (2013) 3: 230; and Jiang et al., *Nucleic Acids Research* (2013) 41(20):e188.

The present disclosure utilizes the CRISPR/Cas9 system that hybridizes with a target sequence in a lineage specific antigen polynucleotide, where the CRISPR/Cas9 system comprises a Cas9 nuclease and an engineered crRNA/tracrRNA (or single guide RNA). CRISPR/Cas9 complex can bind to the lineage specific antigen polynucleotide and allow the cleavage of the antigen polynucleotide, thereby modifying the polynucleotide.

The CRISPR/Cas system of the present disclosure may bind to and/or cleave the region of interest within a cell-surface lineage-specific antigen in a coding or non-coding region, within or adjacent to the gene, such as, for example, a leader sequence, trailer sequence or intron, or within a non-transcribed region, either upstream or downstream of the coding region. The guide RNAs (gRNAs) used in the present disclosure may be designed such that the gRNA directs binding of the Cas9-gRNA complexes to a pre-determined cleavage sites (target site) in a genome. The cleavage sites may be chosen so as to release a fragment that contains a region of unknown sequence, or a region containing a SNP, nucleotide insertion, nucleotide deletion, rearrangement, etc.

Cleavage of a gene region may comprise cleaving one or two strands at the location of the target sequence by the Cas enzyme. In one embodiment, such, cleavage can result in decreased transcription of a target gene. In another embodiment, the cleavage can further comprise repairing the cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein the repair results in an insertion, deletion, or substitution of one or more nucleotides of the target polynucleotide.

The terms "gRNA," "guide RNA" and "CRISPR guide sequence" may be used interchangeably throughout and refer to a nucleic acid comprising a sequence that determines the specificity of a Cas DNA binding protein of a CRISPR/Cas system. A gRNA hybridizes to (complementary to, partially or completely) a target nucleic acid sequence in the genome of a host cell. The gRNA or portion thereof that hybridizes to the target nucleic acid may be between 15-25 nucleotides, 18-22 nucleotides, or 19-21 nucleotides in length. In some embodiments, the gRNA sequence that hybridizes to the target nucleic acid is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In some embodiments, the gRNA sequence that hybridizes to the target nucleic acid is between 10-30, or between 15-25, nucleotides in length.

In addition to a sequence that binds to a target nucleic acid, in some embodiments, the gRNA also comprises a scaffold sequence. Expression of a gRNA encoding both a sequence complementary to a target nucleic acid and scaffold sequence has the dual function of both binding (hybridizing) to the target nucleic acid and recruiting the endonuclease to the target

nucleic acid, which may result in site-specific CRISPR activity. In some embodiments, such a chimeric gRNA may be referred to as a single guide RNA (sgRNA).

As used herein, a "scaffold sequence," also referred to as a tracrRNA, refers to a nucleic acid sequence that recruits a Cas endonuclease to a target nucleic acid bound (hybridized) to a complementary gRNA sequence. Any scaffold sequence that comprises at least one stem loop structure and recruits an endonuclease may be used in the genetic elements and vectors described herein. Exemplary scaffold sequences will be evident to one of skill in the art and can be found, for example, in Jinek, *et al. Science* (2012) 337(6096):816-821, Ran, *et al. Nature Protocols* (2013) 8:2281-2308, PCT Application No. WO2014/093694, and PCT Application No. WO2013/176772.

In some embodiments, the gRNA sequence does not comprises a scaffold sequence and a scaffold sequence is expressed as a separate transcript. In such embodiments, the gRNA sequence further comprises an additional sequence that is complementary to a portion of the scaffold sequence and functions to bind (hybridize) the scaffold sequence and recruit the endonuclease to the target nucleic acid.

In some embodiments, the gRNA sequence is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or at least 100% complementary to a target nucleic acid (see also US Patent 8,697,359, which is incorporated by reference for its teaching of complementarity of a gRNA sequence with a target polynucleotide sequence). It has been demonstrated that mismatches between a CRISPR guide sequence and the target nucleic acid near the 3' end of the target nucleic acid may abolish nuclease cleavage activity (Upadhyay, et al. *Genes Genome Genetics* (2013) 3(12):2233-2238). In some embodiments, the gRNA sequence is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or at least 100% complementary to the 3' end of the target nucleic acid (*e.g.*, the last 5, 6, 7, 8, 9, or 10 nucleotides of the 3' end of the target nucleic acid).

The target nucleic acid is flanked on the 3' side by a protospacer adjacent motif (PAM) that may interact with the endonuclease and be further involved in targeting the endonuclease activity to the target nucleic acid. It is generally thought that the PAM sequence flanking the target nucleic acid depends on the endonuclease and the source from which the endonuclease is derived. For example, for Cas9 endonucleases that are derived from *Streptococcus pyogenes*, the PAM sequence is NGG. For Cas9 endonucleases derived from *Staphylococcus aureus*, the PAM sequence is NNGRRT. For Cas9 endonucleases that are derived from *Neisseria meningitidis*, the PAM sequence is NNNNGATT. For Cas9 endonucleases derived from *Streptococcus thermophilus*, the PAM sequence is NNAGAA.

For Cas9 endonuclease derived from *Treponema denticola*, the PAM sequence is NAAAAC. For a Cpf1 nuclease, the PAM sequence is TTN.

In some embodiments, genetically engineering a cell also comprises introducing a Cas endonuclease into the cell. In some embodiments, the Cas endonuclease and the nucleic acid encoding the gRNA are provided on the same nucleic acid (*e.g.*, a vector). In some embodiments, the Cas endonuclease and the nucleic acid encoding the gRNA are provided on different nucleic acids (*e.g.*, different vectors). Alternatively or in addition, the Cas endonuclease may be provided or introduced into the cell in protein form.

In some embodiments, the Cas endonuclease is a Cas9 enzyme or variant thereof. In some embodiments, the Cas9 endonuclease is derived from *Streptococcus pyogenes*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Streptococcus thermophilus*, or *Treponema denticola*. In some embodiments, the nucleotide sequence encoding the Cas endonuclease may be codon optimized for expression in a host cell. In some embodiments, the endonuclease is a Cas9 homolog or ortholog.

In some embodiments, the nucleotide sequence encoding the Cas9 endonuclease is further modified to alter the activity of the protein. In some embodiments, the Cas9 endonuclease is a catalytically inactive Cas9. For example, dCas9 contains mutations of catalytically active residues (D10 and H840) and does not have nuclease activity. Alternatively or in addition, the Cas9 endonuclease may be fused to another protein or portion thereof. In some embodiments, dCas9 is fused to a repressor domain, such as a KRAB domain. In some embodiments, such dCas9 fusion proteins are used with the constructs described herein for multiplexed gene repression (e.g. CRISPR interference (CRISPRi)). In some embodiments, dCas9 is fused to an activator domain, such as VP64 or VPR. In some embodiments, such dCas9 fusion proteins are used with the constructs described herein for gene activation (*e.g.*, CRISPR activation (CRISPRa)). In some embodiments, dCas9 is fused to an epigenetic modulating domain, such as a histone demethylase domain or a histone acetyltransferase domain. In some embodiments, dCas9 is fused to a LSD1 or p300, or a portion thereof. In some embodiments, the dCas9 fusion is used for CRISPR-based epigenetic modulation. In some embodiments, dCas9 or Cas9 is fused to a Fok1 nuclease domain. In some embodiments, Cas9 or dCas9 fused to a Fok1 nuclease domain is used for genome editing. In some embodiments, Cas9 or dCas9 is fused to a fluorescent protein (*e.g.*, GFP, RFP, mCherry, etc.). In some embodiments, Cas9/dCas9 proteins fused to fluorescent proteins are used for labeling and/or visualization of genomic loci or identifying cells expressing the Cas endonuclease.

Alternatively or in addition, the Cas endonuclease is a Cpf1 nuclease. In some embodiments, the host cell expresses a Cpf1 nuclease derived from *Provetella spp.* or *Francisella spp.* In some embodiments, the nucleotide sequence encoding the Cpf1 nuclease may be codon optimized for expression in a host cell.

In some embodiments, the present disclosure provides compositions and methods for inhibiting a cell-surface lineage-specific antigen in hematopoietic cells using a CRISPR/Cas9 system, wherein guide RNA sequence hybridizes to the nucleotide sequence encoding the cell-surface lineage-specific antigen. In some embodiments, the cell-surface lineage-specific antigen is CD33 and the gRNA hybridizes to a portion of the nucleotide sequence that encodes the CD33 (FIGURE 5). Examples of gRNAs that target CD33 are provided in Table 4, although additional gRNAs may be developed that hybridize to CD33 and can be used in the methods described herein.

Table 4 provides exemplary guide RNA sequences that hybridize or are predicted to hybridize to a portion of CD33.

Table 4: Guide RNA sequences targeting CD33

Name	Guide sequence	Guide position	Score
hCD33-IgC1 referred to herein as "Crispr 1"	ACCTGTCAGGTGAAGTTCGC TGG (SEQ ID NO: 11)	Chr19:+51729270	87
hCD33-IgC3 referred to herein as "Crispr 3"	TGGCCGGGTTCTAGAGTGCC AGG (SEQ ID NO: 28)	Chr19:-51729087	82
hCD33-IgC5 referred to herein as "Crispr 5"	GGCCGGGTTCTAGAGTGCCA GGG (SEQ ID NO: 29)	Chr19:-51729086	81
hCD33 gRNA	CACCGAGGAGTGAGTAGTCC TGG (SEQ ID NO: 30)		
hCD33 gRNA	TCCAGCGAACTTCACCTGAC AGG (SEQ ID NO: 31)		

In some embodiments, it may be desired to further genetically engineer the HSC, particularly allogeneic HSCs, to reduce the graft-versus-host effects. For example, the standard therapy for relapsed AML is hematopoietic stem cell transplantation (HSCT). However, at least one of the limiting factors for successful HSCT is graft-versus-host disease (GVHD), which expression of the cell surface molecule CD45 has been implicated. See, *e.g.*, Van Besie, *Hematology Am. Soc. Hematol Educ Program* (2013)56; Mawad *Curr. Hematol. Malig. Rep.* (2013) 8(2):132. CD45RA and CD45RO are isoforms of CD45 (found on all hematopoietic cells except erythrocytes). In T lymphocytes, CD45RA is expressed on naive cells, while CD45RO is expressed on memory cells. CD45RA T cells have a high potential for reactivity against recipient-specific antigens following HSCT, resulting in GVHD. Thus, there remains a need for efficient and safe AML treatment that would also reduce the possibility of transplant rejection or GVHD. CD45 is a type 1 lineage antigen, since CD45 bearing cells are required for survival but the antigen may be deleted from stem cells using CRISPR.

Taking into account the complications arising due to the development of GvHD following HSCT, the present disclosure also provides compositions and methods for targeting CD45RA. Such compositions and methods are meant to prevent and/or reduce the incidence or extent of GvHD.

Thus, in the case of GVHD, the treatment of the patient can involve the following steps: (1) administering a therapeutically effective amount of a T cell to the patient, where the T cell comprises a nucleic acid sequence encoding a chimeric antigen receptor (CAR) targeting CD45RA lineage specific antigen; and (2) infusing the patient with hematopoietic stem cells, where the hematopoietic cells have reduced expression of CD45RA lineage specific antigen.

Additionally, the present disclosure provides compositions and methods for the combined inhibition of both CD33 and CD45RA lineage specific antigens. Such treatment regimen can involve the following steps: (1) administering a therapeutically effective amount of a T cell to the patient, where the T cell comprises a nucleic acid sequence encoding a chimeric antigen receptor (CAR) targeting both CD33 and CD45RA lineage specific antigens; and (2) infusing or reinfusing the patient with hematopoietic stem cells, either autologous or allogeneic, where the hematopoietic cells have reduced expression of both the CD33 and CD45RA lineage specific antigens.

In some embodiments, the cell-surface lineage-specific antigen CD45RA is also deleted or inhibited in the hematopoietic cells using a CRISPR/Cas9 system. In some embodiments, the gRNA sequence hybridizes to a portion of the nucleotide sequence encoding CD45RA (FIGURE 6). Examples of gRNAs that target CD45RA are provided in Table 5, although additional gRNAs may be developed that hybridize to CD45RA and can be used in the methods described herein.

Table 5 provides exemplary guide RNA sequences that hybridize or are predicted to hybridize to exon 4 or exon 5 of human CD45.

Table 5: Guide RNA sequences targeting CD45

hCD45 Target	Guide RNA
Exon 4	CCAAAGAGTCCGGGGATACT TGG (SEQ ID NO: 9)
	CCAAGTATCCCCGGACTCTT TGG(SEQ ID NO: 32)
	AGCATTATCCAAAGAGTCCG GGG (SEQ ID NO: 33)
	ACTTGGGTGGAAGTATTGTC TGG (SEQ ID NO: 34)
Exon 5	GTTGAGTTTTGCATTGGCGG CGG (SEQ ID NO: 10)
	GTCTGCGAGTCTGCGTGCGT GGG (SEQ ID NO: 35)
	CGTCTGCGAGTCTGCGTGCG TGG (SEQ ID NO: 36)
	GCGAGTCTGCGTGCGTGGGA AGG (SEQ ID NO: 37)

Also provided herein are methods of producing a cell that is deficient in a cell-surface lineage-specific antigen involving providing a cell and introducing into the cell components of a CRISPR Cas system for genome editing. In some embodiments, a nucleic acid that comprises a CRISPR-Cas guide RNA (gRNA) that hybridizes or is predicted to hybridize to a portion of the nucleotide sequence that encodes the lineage-specific cell-surface antigen is introduced into the cell. In some embodiments, the gRNA is introduced into the cell on a vector. In some embodiments, a Cas endonuclease is introduced into the cell. In some emboidments, the Cas endonuclease is introduced into the cell as a nucleic acid encoding a Cas endonuclease. In some embodiments, the gRNA and a nucleotide sequence encoding a Cas endonuclease are introduced into the cell on the same nucleic acid (*e.g.*, the same vector). IN some embodiments, the Cas endonuclease is introduced into the cell in the form of a protein. In some embodiments, the Cas endonuclease and the gRNA are pre-formed *in vitro* and are introduced to the cell in as a complex.

The present disclosure further provides engineered, non-naturally occurring vectors and vector systems, which can encode one or more components of a CRISPR/Cas9 complex, wherein the vector comprises a polynucleotide encoding (i) a (CRISPR)-Cas system guide RNA that hybridizes to the lineage specific antigen sequence and (ii) a Cas9 endonuclease.

Vectors of the present disclosure can drive the expression of one or more sequences in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, *Nature* (1987) 329: 840) and pMT2PC (Kaufman, et al., *EMBO J.* (1987) 6: 187). When used in mammalian cells, the expression vector's control functions are typically provided by one or more regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus, simian virus 40, and others disclosed herein and known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells see, *e.g.*, Chapters 16 and 17 of Sambrook, et al., MOLECULAR CLONING: A LABORATORY MANUAL. 2nd eds., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

The vectors of the present disclosure are capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue- specific regulatory elements are used to express the nucleic acid). Such regulatory elements include promoters that may be tissue specific or cell specific. The term "tissue specific" as it applies to a promoter refers to a promoter that is capable of directing selective expression of a nucleotide sequence of interest to a specific type of tissue (*e.g.*, seeds) in the relative absence of expression of the same nucleotide sequence of interest in a different type of tissue. The term "cell type specific" as applied to a promoter refers to a promoter that is capable of directing selective expression of a nucleotide sequence of interest in a specific type of cell in the relative absence of expression of the same nucleotide sequence of interest in a different type of cell within the same tissue. The term "cell type specific" when applied to a promoter also means a promoter capable of promoting selective expression of a nucleotide sequence of interest in a region within a single tissue. Cell type specificity of a promoter may be assessed using methods well known in the art, *e.g.*, immunohistochemical staining.

Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding CRISPR/Cas9 in mammalian cells or target tissues. Such methods can be used to administer nucleic acids encoding components of a CRISPR-Cas system to cells in culture, or in a host organism. Non-viral vector delivery systems include DNA plasmids, RNA (*e.g.*, a transcript of a vector described herein), naked nucleic acid, and nucleic acid complexed with a delivery vehicle. Viral vector delivery systems include DNA

and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures.

Viral vectors can be administered directly to patients (*in vivo*) or they can be used to manipulate cells *in vitro* or *ex vivo*, where the modified cells may be administered to patients. In one embodiment, the present disclosure utilizes viral based systems including, but not limited to retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Furthermore, the present disclosure provides vectors capable of integration in the host genome, such as retrovirus or lentivirus. Preferably, the vector used for the expression of a CRISPR-Cas system of the present disclosure is a lentiviral vector.

In one embodiment, the disclosure provides for introducing one or more vectors encoding CRISPR-Cas into eukaryotic cell. The cell can be a cancer cell. Alternatively, the cell is a hematopoietic cell, such as a hematopoietic stem cell. Examples of stem cells include pluripotent, multipotent and unipotent stem cells. Examples of pluripotent stem cells include embryonic stem cells, embryonic germ cells, embryonic carcinoma cells and induced pluripotent stem cells (iPSCs). In a preferred embodiment, the disclosure provides introducing CRISPR-Cas9 into a hematopoietic stem cell.

The vectors of the present disclosure are delivered to the eukaryotic cell in a subject. Modification of the eukaryotic cells via CRISPR/Cas9 system can takes place in a cell culture, where the method comprises isolating the eukaryotic cell from a subject prior to the modification. In some embodiments, the method further comprises returning said eukaryotic cell and/or cells derived therefrom to the subject.

Combined Therapy

As described herein, agents comprising an antigen-binding fragment that binds to a cell-surface lineage-specific antigen may be administered to a subject in combination with hematopoietic cells that are deficient for the cell-surface lineage-specific antigen. As used herein, "subject," "individual," and "patient" are used interchangeably, and refer to a vertebrate, preferably a mammal such as a human. Mammals include, but are not limited to, human primates, non-human primates or murine, bovine, equine, canine or feline species. In some embodiments, the subject is a human patient having a hematopoietic malignancy.

In some embodiments, the agents and/or the hematopoietic cells may be mixed with a pharmaceutically acceptable carrier to form a pharmaceutical composition, which is also within the scope of the present disclosure.

To perform the methods described herein, an effective amount of the agent comprising an antigen-binding fragment that binds to a cell-surface lineage-specific antigen and an effective amount of hematopoietic cells can be co-administered to a subject in need of the treatment. As used herein the term "effective amount" may be used interchangeably with the term "therapeutically effective amount" and refers to that quantity of an agent, cell population, or pharmaceutical composition (*e.g.*, a composition comprising agents and/or hematopoietic cells) that is sufficient to result in a desired activity upon administration to a subject in need thereof. Within the context of the present disclosure, the term "effective amount" refers to that quantity of a compound, cell population, or pharmaceutical composition that is sufficient to delay the manifestation, arrest the progression, relieve or alleviate at least one symptom of a disorder treated by the methods of the present disclosure. Note that when a combination of active ingredients is administered the effective amount of the combination may or may not include amounts of each ingredient that would have been effective if administered individually.

Effective amounts vary, as recognized by those skilled in the art, depending on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size, gender and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. In some embodiments, the effective amount alleviates, relieves, ameliorates, improves, reduces the symptoms, or delays the progression of any disease or disorder in the subject. In some embodiments, the subject is a human. In some embodiments, the subject is a human patient having a hematopoietic malignancy.

As described herein, the hematopoietic cells and/or immune cells expressing chimeric receptors may be autologous to the subject, *i.e.*, the cells are obtained from the subject in need of the treatment, genetically engineered to be deficient for expression of the cell-surface lineage-specific antigen or for expression of the chimeric receptor constructs, and then administered to the same subject. Administration of autologous cells to a subject may result in reduced rejection of the host cells as compared to administration of non-autologous cells. Alternatively, the host cells are allogeneic cells, i.e., the cells are obtained from a first subject, genetically engineered to be deficient for expression of the cell-surface lineage-specific antigen or for expression of the chimeric receptor constructs, and administered to a second subject that is different from the first subject but of the same species. For example,

allogeneic immune cells may be derived from a human donor and administered to a human recipient who is different from the donor.

In some embodiments, the immune cells and/or hematopoietic cells are allogeneic cells and have been further genetically engineered to reduced graft-versus-host disease. For example, as described herein, the hematopoietic stem cells may be genetically engineered (*e.g.*, using genome editing) to have reduced expression of CD45RA.

In some embodiments, the immune cells expressing any of the chimeric receptors described herein are administered to a subject in an amount effective in to reduce the number of target cells (*e.g.*, cancer cells) by least 20%, *e.g.*, 50%, 80%, 100%, 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold or more.

A typical amount of cells, *i.e.*, immune cells or hematopoietic cells, administered to a mammal (*e.g.*, a human) can be, for example, in the range of one million to 100 billion cells; however, amounts below or above this exemplary range are also within the scope of the present disclosure. For example, the daily dose of cells can be about 1 million to about 50 billion cells (*e.g.*, about 5 million cells, about 25 million cells, about 500 million cells, about 1 billion cells, about 5 billion cells, about 20 billion cells, about 30 billion cells, about 40 billion cells, or a range defined by any two of the foregoing values), preferably about 10 million to about 100 billion cells (*e.g.*, about 20 million cells, about 30 million cells, about 40 million cells, about 60 million cells, about 70 million cells, about 80 million cells, about 90 million cells, about 10 billion cells, about 25 billion cells, about 50 billion cells, about 75 billion cells, about 90 billion cells, or a range defined by any two of the foregoing values), more preferably about 100 million cells to about 50 billion cells (*e.g.*, about 120 million cells, about 250 million cells, about 350 million cells, about 450 million cells, about 650 million cells, about 800 million cells, about 900 million cells, about 3 billion cells, about 30 billion cells, about 45 billion cells, or a range defined by any two of the foregoing values).

In one embodiment, the chimeric receptor (*e.g.*, a nucleic acid encoding the chimeric receptor) is introduced into an immune cell, and the subject (*e.g.*, human patient) receives an initial administration or dose of the immune cells expressing the chimeric receptor. One or more subsequent administrations of the agent (*e.g.*, immune cells expressing the chimeric receptor) may be provided to the patient at intervals of 15 days, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 days after the previous administration. More than one dose of the agent can be administered to the subject per week, *e.g.*, 2, 3, 4, or more administrations of the agent. The subject may receive more than one doses of the agent (*e.g.*, an immune cell expressing a chimeric receptor) per week, followed by a week of no administration of the agent, and

finally followed by one or more additional doses of the agent (*e.g.*, more than one administration of immune cells expressing a chimeric receptor per week). The immune cells expressing a chimeric receptor may be administered every other day for 3 administrations per week for two, three, four, five, six, seven, eight or more weeks.

In the context of the present disclosure insofar as it relates to any of the disease conditions recited herein, the terms "treat," "treatment," and the like mean to relieve or alleviate at least one symptom associated with such condition, or to slow or reverse the progression of such condition. Within the meaning of the present disclosure, the term "treat" also denotes to arrest, delay the onset (i.e., the period prior to clinical manifestation of a disease) and/or reduce the risk of developing or worsening a disease. For example, in connection with cancer the term "treat" may mean eliminate or reduce a patient's tumor burden, or prevent, delay or inhibit metastasis, etc.

In some embodiments, an agent comprising an antigen-binding fragment that binds a cell-surface lineage-specific antigen and a population of hematopoietic cells deficient in the cell-surface lineage-specific antigen. Accordingly, in such therapeutic methods, the agent recognizes (binds) a target cell expressing the cell-surface lineage-specific antigen for targeting killing. The hematopoietic cells that are deficient in the antigen allow for repopulation of a cell type that is targeted by the agent. In some embodiments, the treatment of the patient can involve the following steps: (1) administering a therapeutically effective amount of an agent targeting a cell-surface lineage-specific antigen to the patient and (2) infusing or reinfusing the patient with hematopoietic stem cells, either autologous or allogenic, where the hematopoietic cells have reduced expression of a lineage specific disease-associated antigen. In some embodiments, the treatment of the patient can involve the following steps: (1) administering a therapeutically effective amount of an immune cell expressing a chimeric receptor to the patient, wherein the immune cell comprises a nucleic acid sequence encoding a chimeric receptor that binds a cell-surface lineage-specific, disease-associated antigen; and (2) infusing or reinfusing the patient with hematopoietic cells (*e.g.*, hematopoietic stem cells), either autologous or allogenic, where the hematopoietic cells have reduced expression of a lineage specific disease-associated antigen.

The efficacy of the therapeutic methods using a an agent comprising an antigen-binding fragment that binds a cell-surface lineage-specific antigen and a population of hematopoietic cells deficient in the cell-surface lineage-specific antigen may be assessed by any method known in the art and would be evident to a skilled medical professional. For example, the efficacy of the therapy may be assessed by survival of the subject or cancer

burden in the subject or tissue or sample thereof. In some embodiments, the efficacy of the therapy is assessed by quantifying the number of cells belonging to a particular population or lineage of cells. In some embodiments, the efficacy of the therapy is assessed by quantifying the number of cells presenting the cell-surface lineage-specific antigen.

In some embodiments, the agent comprising an antigen-binding fragment that binds to the cell-surface lineage-specific antigen and the population of hematopoietic cells IS administered concomitantly.

In some embodiments, the agent comprising an antigen-binding fragment that binds a cell-surface lineage-specific antigen (*e.g.*, immune cells expressing a chimeric receptor as described herein) is administered prior to administration of the hematopoietic cells. In some embodiments, the agent comprising an antigen-binding fragment that binds a cell-surface lineage-specific antigen (*e.g.*, immune cells expressing a chimeric receptor as described herein) is administered at least about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 3 months, 4 months, 5 months, 6 months or more prior to administration of the hematopoietic cells.

In some embodiments, the hematopoietic cells are administered prior to the agent comprising an antigen-binding fragment that binds a cell-surface lineage-specific antigen (*e.g.*, immune cells expressing a chimeric receptor as described herein). In some embodiments, the population of hematopoietic cells is administered at least about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 3 months, 4 months, 5 months, 6 months or more prior to administration of the agent comprising an antigen-binding fragment that binds to the cell-surface lineage-specific antigen.

In some embodiments, the agent targeting the cell-surface lineage-specific antigen and the population of hematopoietic cells are administered at substantially the same time. In some embodiments, agent targeting the cell-surface lineage-specific antigen is administered and the patient is assessed for a period of time, after which the population of hematopoietic cells is administered. In some embodiments, the population of hematopoietic cells is administered and the patient is assessed for a period of time, after which agent targeting the cell-surface lineage-specific antigen is administered.

Also within the scope of the present disclosure are multiple administrations (*e.g.*, doses) of the agents and/or populations of hematopoietic cells. In some embodiments, the agents and/or populations of hematopoietic cells are administered to the subject once. In

some embodiments, agents and/or populations of hematopoietic cells are administered to the subject more than once (*e.g.*, at least 2, 3, 4, 5, or more times). In some embodiments, the agents and/or populations of hematopoietic cells are administered to the subject at a regular interval, *e.g.*, every six months.

In some embodiments, the subject is a human subject having a hematopoietic malignancy. As used herein a hematopoietic malignancy refers to a malignant abnormality involving hematopoietic cells (*e.g.*, blood cells, including progenitor and stem cells). Examples of hematopoietic malignancies include, without limitation, Hodgkin's lymphoma, non-Hodgkin's lymphoma, leukemia, or multiple myeloma. Leukemias include acute myeloid leukaemia, acute lymphoid leukemia, chronic myelogenous leukaemia, acute lymphoblastic leukemia or chronic lymphoblastic leukemia, and chronic lymphoid leukemia.

In some embodiments, the leukemia is acute myeloid leukaemia (AML). AML is characterized as a heterogeneous, clonal, neoplastic disease that originates from transformed cells that have progressively acquired critical genetic changes that disrupt key differentiation and growth-regulatory pathways. (Dohner et al., *NEJM,* (2015) 373:1136). CD33 glycoprotein is expressed on the majority of myeloid leukemia cells as well as on normal myeloid and monocytic precursors and has been considered to be an attractive target for AML therapy (Laszlo et al., *Blood Rev.* (2014) 28(4):143-53). While clinical trials using anti CD33 monoclonal antibody based therapy have shown improved survival in a subset of AML patients when combined with standard chemotherapy, these effects were also accompanied by safety and efficacy concerns.

Other efforts aimed at targeting AML cells have involved the generation of T cells expressing chimeric antigen receptors (CARs) that selectively target CD33 in AML. Buckley et al., *Curr. Hematol. Malig. Rep.* (2):65 (2015). However, the data is limited and there are uncertainties about how effective (whether all targeted cells are eliminated) this approach may be in treating the patient. Additionally, since myeloid lineage cells are indispensable for life, depleting a subject of myeloid lineage cells could have detrimental effects on survival of the patient. The present disclosure aims at, at least in part, solving such problems associated with AML treatment.

Alternatively or in addition, the methods described herein may be used to treat non-hematopoietic cancers, including without limitation, lung cancer, ear, nose and throat cancer, colon cancer, melanoma, pancreatic cancer, mammary cancer, prostate cancer, breast cancer, ovarian cancer, basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; breast cancer; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer;

cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer; intra-epithelial neoplasm; kidney cancer; larynx cancer; liver cancer; fibroma, neuroblastoma; oral cavity cancer (e.g., lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; renal cancer; cancer of the respiratory system; sarcoma; skin cancer; stomach cancer; testicular cancer; thyroid cancer; uterine cancer; cancer of the urinary system, as well as other carcinomas and sarcomas.

Carcinomas are cancers of epithelial origin. Carcinomas intended for treatment with the methods of the present disclosure include, but are not limited to, acinar carcinoma, acinous carcinoma, alveolar adenocarcinoma (also called adenocystic carcinoma, adenomyoepithelioma, cribriform carcinoma and cylindroma), carcinoma adenomatosum, adenocarcinoma, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma (also called bronchiolar carcinoma, alveolar cell tumor and pulmonary adenomatosis), basal cell carcinoma, carcinoma basocellulare (also called basaloma, or basiloma, and hair matrix carcinoma), basaloid carcinoma, basosquamous cell carcinoma, breast carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriform carcinoma, cholangiocellular carcinoma (also called cholangioma and cholangiocarcinoma), chorionic carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epibulbar carcinoma, epidermoid carcinoma, carcinoma epitheliale adenoides, carcinoma exulcere, carcinoma fibrosum, gelatiniform carcinoma, gelatinous carcinoma, giant cell carcinoma, gigantocellulare, glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, hematoid carcinoma, hepatocellular carcinoma (also called hepatoma, malignant hepatoma and hepatocarcinoma), Huirthle cell carcinoma, hyaline carcinoma, hypernephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma mastitoides, carcinoma medullare, medullary carcinoma, carcinoma melanodes, melanotic carcinoma, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, nasopharyngeal carcinoma, carcinoma nigrum, oat cell carcinoma, carcinoma ossificans, osteoid carcinoma, ovarian carcinoma, papillary carcinoma, periportal carcinoma, preinvasive carcinoma, prostate carcinoma, renal cell carcinoma of kidney (also

called adenocarcinoma of kidney and hypemephoroid carcinoma), reserve cell carcinoma, carcinoma sarcomatodes, scheinderian carcinoma, scirrhous carcinoma, carcinoma scroti, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberous carcinoma, verrucous carcinoma, carcinoma vilosum. In preferred embodiments, the methods of the present disclosure are used to treat subjects having cancer of the breast, cervix, ovary, prostate, lung, colon and rectum, pancreas, stomach or kidney.

Sarcomas are mesenchymal neoplasms that arise in bone and soft tissues. Different types of sarcomas are recognized and these include: liposarcomas (including myxoid liposarcomas and pleiomorphic liposarcomas), leiomyosarcomas, rhabdomyosarcomas, malignant peripheral nerve sheath tumors (also called malignant schwannomas, neurofibrosarcomas, or neurogenic sarcomas), Ewing's tumors (including Ewing's sarcoma of bone, extraskeletal (i.e., non-bone) Ewing's sarcoma, and primitive neuroectodermal tumor [PNET]), synovial sarcoma, angiosarcomas, hemangiosarcomas, lymphangiosarcomas, Kaposi's sarcoma, hemangioendothelioma, fibrosarcoma, desmoid tumor (also called aggressive fibromatosis), dermatofibrosarcoma protuberans (DFSP), malignant fibrous histiocytoma (MFH), hemangiopericytoma, malignant mesenchymoma, alveolar soft-part sarcoma, epithelioid sarcoma, clear cell sarcoma, desmoplastic small cell tumor, gastrointestinal stromal tumor (GIST) (also known as GI stromal sarcoma), osteosarcoma (also known as osteogenic sarcoma)-skeletal and extraskeletal, and chondrosarcoma.

In some embodiments, the cancer to be treated can be a refractory cancers. A "refractory cancer," as used herein, is a cancer that is resistant to the standard of care prescribed. These cancers may appear initially responsive to a treatment (and then recur), or they may be completely non-responsive to the treatment. The ordinary standard of care will vary depending upon the cancer type, and the degree of progression in the subject. It may be a chemotherapy, or surgery, or radiation, or a combination thereof. Those of ordinary skill in the art are aware of such standards of care. Subjects being treated according to the present disclosure for a refractory cancer therefore may have already been exposed to another treatment for their cancer. Alternatively, if the cancer is likely to be refractory (e.g., given an analysis of the cancer cells or history of the subject), then the subject may not have already been exposed to another treatment. Examples of refractory cancers include, but are not

limited to, leukemia, melanomas, renal cell carcinomas, colon cancer, liver (hepatic) cancers, pancreatic cancer, Non-Hodgkin's lymphoma and lung cancer.

Any of the immune cells expressing chimeric receptors described herein may be administered in a pharmaceutically acceptable carrier or excipient as a pharmaceutical composition.

The phrase "pharmaceutically acceptable," as used in connection with compositions and/or cells of the present disclosure, refers to molecular entities and other ingredients of such compositions that are physiologically tolerable and do not typically produce untoward reactions when administered to a mammal (*e.g.*, a human). Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in mammals, and more particularly in humans. "Acceptable" means that the carrier is compatible with the active ingredient of the composition (*e.g.*, the nucleic acids, vectors, cells, or therapeutic antibodies) and does not negatively affect the subject to which the composition(s) are administered. Any of the pharmaceutical compositions and/or cells to be used in the present methods can comprise pharmaceutically acceptable carriers, excipients, or stabilizers in the form of lyophilized formations or aqueous solutions.

Pharmaceutically acceptable carriers, including buffers, are well known in the art, and may comprise phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives; low molecular weight polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; amino acids; hydrophobic polymers; monosaccharides; disaccharides; and other carbohydrates; metal complexes; and/or non-ionic surfactants. See, *e.g.* Remington: *The Science and Practice of Pharmacy* 20th Ed. (2000) Lippincott Williams and Wilkins, Ed. K. E. Hoover.

Kits for Therapeutic Uses

Also within the scope of the present disclosure are kits for use of the agents targeting cell-surface lineage-specific antigens in combination with populations of hematopoietic cells that are deficient in the cell-surface lineage-specific antigen. Such kits may include one or more containers comprising a first pharmaceutical composition that comprises any agent comprising an antigen-binding fragment that binds a cell-surface lineage-specific antigen (*e.g.*, immune cells expressing chimeric receptors described herein), and a pharmaceutically acceptable carrier, and a second pharmaceutical composition that comprises a population of

hematopoietic cells that are deficient in the cell-surface lineage-specific antigen (*e.g.*, a hematopoietic stem cell) and a pharmaceutically acceptable carrier.

In some embodiments, the kit can comprise instructions for use in any of the methods described herein. The included instructions can comprise a description of administration of the first and second pharmaceutical compositions to a subject to achieve the intended activity in a subject. The kit may further comprise a description of selecting a subject suitable for treatment based on identifying whether the subject is in need of the treatment. In some embodiments, the instructions comprise a description of administering the first and second pharmaceutical compositions to a subject who is in need of the treatment.

The instructions relating to the use of the agents targeting cell-surface lineage-specific antigens and the first and second pharmaceutical compositions described herein generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the disclosure are typically written instructions on a label or package insert. The label or package insert indicates that the pharmaceutical compositions are used for treating, delaying the onset, and/or alleviating a disease or disorder in a subject.

The kits provided herein are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Also contemplated are packages for use in combination with a specific device, such as an inhaler, nasal administration device, or an infusion device. A kit may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The container may also have a sterile access port. At least one active agent in the pharmaceutical composition is a chimeric receptor variants as described herein.

Kits optionally may provide additional components such as buffers and interpretive information. Normally, the kit comprises a container and a label or package insert(s) on or associated with the container. In some embodiment, the disclosure provides articles of manufacture comprising contents of the kits described above.

General techniques

The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of

the art. Such techniques are explained fully in the literature, such as *Molecular Cloning: A Laboratory Manual,* second edition (Sambrook, et al., 1989) Cold Spring Harbor Press; *Oligonucleotide Synthesis* (M. J. Gait, ed. 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J. E. Cellis, ed., 1989) Academic Press; Animal Cell Culture (R. I. Freshney, ed. 1987); Introuction to Cell and Tissue Culture (J. P. Mather and P. E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J. B. Griffiths, and D. G. Newell, eds. 1993-8) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D. M. Weir and C. C. Blackwell, eds.): Gene Transfer Vectors for Mammalian Cells (J. M. Miller and M. P. Calos, eds., 1987); Current Protocols in Molecular Biology (F. M. Ausubel, et al. eds. 1987); PCR: The Polymerase Chain Reaction, (Mullis, et al., eds. 1994); Current Protocols in Immunology (J. E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C. A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practice approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, eds. Harwood Academic Publishers, 1995); *DNA Cloning: A practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds.(1985»; *Transcription and Translation* (B.D. Hames & S.J. Higgins, eds. (1984»; *Animal Cell Culture* (R.I. Freshney, ed. (1986»; *Immobilized Cells and Enzymes* (IRL Press, (1986»; and B. Perbal, *A practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.).

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present disclosure to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

EXAMPLE 1: *In vitro* Deletion of CD33 in a Human Leukemic Cell Line

In order to test the ability of CRSPR-Cas9 system to target CD33 *in vitro*, human leukemic cells K-562 were co-transfected using Neon™ (Thermo Fisher Scientific) with Cas9-GFP (PX458, *S. pyogenes*) and a guide RNA containing NGG PAM sequence (FIGURE 4) where guide RNA was designed to target hCD33 genomic sequence. 48 hours

post-transfection, cells expressing Cas9 were identified and isolated using FACS sorting for GFP. Cells were then incubated for 96 hours and tested for CD33 expression by flow cytometry (FIGURE 5). Flow cytometry plots using an anti-CD33 antibody show CD33 expression by the K-562 cells before (top plot) and after (bottom plot) delivery of Cas9 vector and guide RNA. As shown in FIGURE 5, 98% of the cells lacked the CD33 expression following transfection.

This example demonstrates the efficient deletion of CD33 using CRISPR-Cas9 system in human leukemic cells.

EXAMPLE 2: *In vitro* Deletion of CD45 in Human Leukemic Cell Lines

The CRISPR-Cas9 system was used to target CD45RA *in vitro*. Briefly, TIB-67 reticulum cell sarcoma mouse macrophage-like cells were co-transfected using Neon™ reagent (Thermo Fisher Scientific) with Cas9-GFP (PX458, *S. pyogenes*) and CRISPRs gRNAs (containing the "NGG" PAM sequence) targeting hCD45RA genomic sequence. 48 hours post-transfection, cells expressing CRISPR-Cas9 system were identified and isolated using FACS sorting for GFP. Cells were then incubated for 96 hours and tested for CD45RA expression (FIGURE 6). Flow cytometry plots using CD45RA antibody show CD45RA expression before (top plot) and after (bottom plot) delivery of Cas9 vector and guide RNA.

Similar to Example 1, where CD33 expression was successfully reduced in leukemic cells, findings in this Example indicate efficient targeting of CD45RA using the CRISPR-Cas9 system.

EXAMPLE 3: Targeting Cell-surface Lineage-specific CD33 in Acute Myeloid Leukemia (AML)

The present example encompasses targeting of the CD33 antigen in AML. The specific steps of the example are outlined in Table 6.

Table 6. Outline of the Experimental Design

I. Autologous CD33 targeted (CAR) T- cell therapy	1.Generation of anti-CD33 CAR constructs 2. Isolation of $CD8^+$ T Cells from a Patient 3. Preparation of anti CD33 CAR T Cells 4. Reinfusion of CD33 CAR T cells into a Patient
II. Autologous Hematopoietic Stem Cell Transplant Using $CD34^+CD33^-$ Cells	1. Isolation of Hematopoietic Stem Cells 2. CRISPR-Cas9 Plasmid Targeting CD33 3. Generation of $CD34^+CD33^-$ cells via CRISPR-CAS System

	4. Reinfusion of $CD34^+CD33^-$ cells into a Patient
III. Continued treatment of a patient with a CD33 antibody attached to a toxin (immunotoxin)	

I. CD33-targeted chimeric antigen receptor (CAR) T-cell therapy

A. Generation of anti-CD33 CAR constructs

The chimeric antigen receptors targeting CD33 described herein may consist of the following components in order from 5′ to 3′: pHIV-Zsgreen lentiviral backbone (www.addgene.org/18121/), peptide signal, the CD33 scFv, the hinge, transmembrane regions of the CD28 molecule, the intracellular domain of CD28, and the signaling domain of TCR-ζ molecule.

Initially, peptide signal, anti-CD33 light chain (SEQ ID NO: 1), the flexible linker and the anti-CD33 heavy chain (SEQ ID. NO. 2) are cloned into the EcoRI site of pHIV-Zsgreen, with an optimal Kozak sequence.

The nucleic acid sequences of an exemplary chimeric receptors that binds CD33 with the basic structure of Light chain- linker –Heavy chain- Hinge-CD28/ICOS –CD3ζ is provided below.

Part 1: Light chain- linker –Heavy chain (SEQ ID NO: 16): The Kozak start site is shown in boldface. The peptide signal L1 is shown in italic. The anti-CD33 light chain and heavy chain are shown in bold and italics, separated by a linker.

ggtgtcgtgagcggccgctgaactg**GCCACC**ATG*GACATGAGGGTCCCTGCTCAGCTCCT*
*GGGGCTCCTGCTGCTCTGGCTCTCAGGTGCCAGATGT****GAGATCGTGCTGACCCAGAGCCC***
CGGCAGCCTGGCCGTGAGCCCCGGCAAGAGGGTGACCATGAGCTGCAAGAGCAGCCAGAG
CGTGTTCTTCAGCAGCAGCCAGAAGAACTACCTGGCCTGGTACCAGCAGATCCCCGGCCA
GAGCCCCAGGCTGCTGATCTACTGGGCCAGCACCAGGGAGAGCGGCGTGCCCGACAGGTT
CACCGGCAGCGGCAGCGGCAGCGGCACCGACTTCACCCTGACCATCAGCAGCGTGCAGCC
CGAGGACCTGGCCATCTACTACTGCCACCAGTACCTGAGCAGCAGGACCTTCGGCCAGGG
CACCAAGCTGGAGATCAAGAGGGGCAGCACCAGCGGCAGCGGCAAGCCCGGCAGCGGCGA
GGGCAGCACCAAGGGC***CAGGTGCAGCTGCAGCAGCCCGGCGCCGAGGTGGTGAAGCCCGG***
CGCCAGCGTGAAGATGAGCTGCAAGGCCAGCGGCTACACCTTCACCAGCTACTACATCCA
CTGGATCAAGCAGACCCCCGGCCAGGGCCTGGAGTGGGTGGGCGTGATCTACCCCGGCAA
CGACGACATCAGCTACAACCAGAAGTTCCAGGGCAAGGCCACCCTGACCGCCGACAAGAG
CAGCACCACCGCCTACATGCAGCTGAGCAGCCTGACCAGCGAGGACAGCGCCGTGTACTA

CTGCGCCAGGGAGGTGAGGCTGAGGTACTTCGACGTGTGGGGCCAGGGCACCACCGTGAC CGTGAGCAGC

Part 2: Hinge-CD28/ICOS –CD3ζ NotI restriction enzyme recognition sites are shown in capitalization. The translational stop site is in boldface. The BamHI restriction cleavage site is shown in underline.

CD28 costimulatory domain (SEQ ID NO: 17)

GCGGCCGCAattgaagttatgtatcctcctccttacctagacaatgagaagagcaatgga
accattatccatgtgaaagggaaacacctttgtccaagtccccctatttccccggaccttct
aagccctttttgggtgctggtggtggttggtggagtcctggcttgctatagcttgctagta
acagtggcctttattatttctgggtgaggagtaagaggagcaggctcctgcacagtgac
tacatgaacatgactccccgccgccccgggcccAccccgcaagcattaccagccctatgcc
ccaccacgcgacttcgcagcctatcgctccagagtgaagttcagcaggagcgcagacgcc
ccccgcgtaccagcagggccagaaccagctctataacgagctcaatctaggacgaagagag
gagtacgatgttttggacaagagacgtggccgggaccctgagatgggggcgaaagccgaga
aggaagaaccctcaggaaggcctgtacaatgaactgcagaaagataagatggcggaggcc
tacagtgagattgggatgaaaggcgagcgccggaggggcaaggggcacgatggcctttac
cagggtctcagtacagccaccaaggacacctacgacgcccttcacatgcaggccctgccc
cctgcTAAcgcccctctccctcccccccccctaa

ICOS costimulatory domain (SEQ ID NO: 18)

GCGGCCGCActatcaatttttgatcctcctcctttaaagtaactcttacaggaggatat
ttgcatatttatgaatcacaactttgttgccagctgaagttctggttacccataggatgt
gcagcctttgttgtagtctgcattttgggatgcatacttatttgttggcttacaaaaaag
aagtattcatccagtgtgcacgaccctaacggtgaatacatgttcatgagagcagtgaac
acagccaaaaaatctagactcacagatgtgaccctaagagtgaagttcagcaggagcgca
gacgccccgcgtaccagcagggccagaaccagctctataacgagctcaatctaggacga
agagaggagtacgatgttttggacaagagacgtggccgggaccctgagatgggggaaag
ccgagaaggaagaaccctcaggaaggcctgtacaatgaactgcagaaagataagatggcg
gaggcctacagtgagattgggatgaaaggcgagcgccggaggggcaaggggcacgatggc
ctttaccagggtctcagtacagccaccaaggacacctacgacgcccttcacatgcaggcc
ctgcccccctcgcTAAcgcccctctccctcccccccccctaa

Fusion (hybrid) CD28 and ICOS costimulatory domain (SEQ ID NO: 19)

GCGGCCGCAattgaagttatgtatcctcctccttacctagacaatgagaagagcaatgga
accattatccatgtgaaagggaaacacctttgtccaagtccccctatttccccggaccttct
aagccctttttgggtgctggtggtggttggtggagtcctggcttgctatagcttgctagta
acagtggcctttattatttctgggtgaggagtaagaggagcaggctcctgcacagtgac
tacatgttcatgagagcagtgaacacagccaaaaaatctagactcacagatgtgaccccta
agagtgaagttcagcaggagcgcagacgcccccgcgtaccagcagggccagaaccagctc
tataacgagctcaatctaggacgaagagaggagtacgatgttttggacaagagacgtggc
cgggaccctgagatgggggcgaaagccgagaaggaagaaccctcaggaaggcctgtacaat
gaactgcagaaagataagatggcggaggcctacagtgagattgggatgaaaggcgagcgc
cggaggggcaaggggcacgatggcctttaccagggtctcagtacagccaccaaggacacc

tacgacgccctttcacatgcaggccctgccccctcgcTAAcgcccctctccctcccccccc
cctaa

In the next step, the hinge region, CD28 domain (SEQ ID NO: 3) and a cytoplasmic component of TCR-ζ are cloned into the NotI and BamHI sites of pHIV-Zsgreen (already containing the peptide signal and the CD33 scFv. Alternatively, CD28 domain can be substituted by ICOS domain (SEQ ID NO: 4).

In addition to CD28 and ICOS domains, a fusion domain comprising fragments of CD28 and ICOS intracellular signaling domains will be engineered (SEQ ID NO: 5) and used to generate additional chimeric receptors. Such configuration, where the chimeric receptor comprises an antigen-binding fragment, an anti-CD33 light chain variable region, a linker, an anti-CD33 heavy chain variable region, CD28/ICOS hybrid region (including a TM region of CD28), and signaling domain of TCR-ζ molecule.

Example amino acid sequences of components that may be used to generate the chimeric receptors are provided herein, such as CD28 domain (SEQ ID NO: 6), ICOS domain (SEQ ID NO: 7), CD28/ICOS hybrid domain (SEQ ID NO: 8), and TCR-ζ are provided herein. Alternatively, the chimeric receptor may be generated as well (Section B.)

B. Alternative Method for Generation of anti-CD33 CAR constructs

Schematics of example chimeric receptors are presented in FIGURE 7, panels A-D. The chimeric receptor will be generated using an extracellular humanized scFv recognizing the CD33 antigen, linked to an extracellular CD8 hinge region, a transmembrane and cytoplasmic signaling domain, and a CD3 ζ-signaling chain (FIGURE 7, panel B). DNA encoding the anti-CD33 chimeric receptor will be generated by using a humanized scFv (Essand et al., *J Intern Med*_(2013) 273(2):166). Alternatives include a CAR T cell that contains OX-1 or 41-BB in place of CD28 or CD28/OX1 or CD28/4-1-B-B hybrids (FIGURE 7, panels C and D).

In order to generate the anti-CD33 scFV sequence, the coding regions of the heavy and light chains of the variable regions of the anti-CD33 antibody described above (SEQ ID NOs: 1 and 2) will be amplified with specific primers and cloned into a pHIV-Zsgreen vector for expression in cells. To evaluate the binding strength of the scFv (single chain variable fragments) to the target antigen, the scFv will be expressed in Hek293T cells. For this purpose, the vector (pHIV-Zsgreen containing the coding areas) will be transformed into *E. coli* Top10F bacteria and the plasmids prepared. The obtained expression vectors that code for the scFv antibodies will be introduced by transfection into Hek293T cells. After culturing

the transfected cells for five days, the supernatant will be removed and the antibodies purified..

The resulting antibodies can be humanized using framework substitutions by protocols known in the art. See, for example, one such protocol is provided by BioAtla (San Diego), where synthetic CDR encoding fragment libraries derived from a template antibody are ligated to human framework region encoding fragments from a human framework pool limited to germline sequences from a functionally expressed antibodies (bioatla.com/applications/express-humanization/).

Affinity maturation may be performed in order to improve antigen binding affinity. This can be accomplished using general techniques known in the art, such as phage display (Schier R., *J. Mol. Biol* (1996), 263:551). The variants can be screened-for their biological activity (*e.g.*, binding affinity) using for example Biacore analysis. In order to identify hypervariable region residues which would be good candidates for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Additionally, combinatorial libraries described by can also be used for improving the affinity of the antibodies (Rajpal et al., *PNAS* (2005) 102(24): 8466). Alternatively, BioAtla has developed a platform for the rapid and efficient affinity maturation of antibodies, which can also be utilized for the purposes of antibody optimization (bioatla.com/applications/functional-maturation/).

(C) Assembly of CAR construct

Next, the anti-CD33 scFv will be linked to an extracellular CD8 hinge region, a transmembrane and cytoplasmic CD28 signaling domain, and a CD3 ζ-signaling chain. Briefly, primers specific for anti-CD33 scFv sequence will be used to amplify the scFv as described above. Plasmid (pUN1-CD8). (www.invivogen.com/puno-cd8a) carrying the complete human CD8 coding sequence will be used to amplify CD8 hinge and transmembrane domains (amino acids 135–205). CD3 ζ fragment will be amplified from the Invivogen plasmid pORF9-hCD247a (http://www.invivogen.com/PDF/pORF9-hCD247a_10E26v06.pdf) carrying the complete human CD3ζ coding sequence. Finally, the CD28 (amino acids 153-220, corresponding to TM and signaling domains of CD28) will be amplified from cDNA generated using RNA collected from activated T cells by Trizol method. Fragments containing anti-CD33-scFv-CD8-hinge+TM-CD28-CD3ζ will be assembled using splice overlap extension (SOE) PCR. The resulting PCR fragment will then be cloned into pELPS lentiviral vector. pELPS is a derivative of the third-generation

lentiviral vector pRRL-SIN-CMV-eGFP-WPRE in which the CMV promoter was replaced with the EF-1α promoter and the central polypurine tract of HIV was inserted 5′ of the promoter (Milone et al., *Mol Ther*. (2009) (8): 1453, Porter et al., *NEJM* (2011) (8):725). All constructs will be verified by sequencing.

Alternatively, CARs containing ICOS, CD27, 41BB, or OX-40 signaling domain instead of CD28 domain will be generated, introduced into T-cells and tested for the ability to eradicate CD33 positive cells (FIGURE 7, panel C). The generation of "third-generation" chimeric receptors are also contemplated (FIGURE 7, panel D), which combine multiple signaling domains, such as CD3z-CD28-41BB or CD3z-CD28-OX40, to further augment potency (Sadelain et al., *Cancer Discov.*(2013) 4:388).

(D) Anti-CD33 CAR T Cell Preparation

Primary human $CD8^+$ T cells will be isolated from patients' peripheral blood by immunomagnetic separation (Miltenyi Biotec). T cells will be cultured in complete media (RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, 10mM HEPES) and stimulated with anti-CD3 and anti-CD28 mAbs–coated beads (Invitrogen) as previously described (Levine et al., *J. Immunol.* (1997) 159(12):5921).

A packaging cell line will be used to generate the viral vector, that is able to transduce target cells and contains the anti-CD33 chimeric receptors. To generate lentiviral particles, CARs generated in section (1) of this Example will be transfected into immortalized normal fetal renal 293T packaging cells together with Cells will be cultured with high glucose DMEM, including 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. 48-72 hours post-transfection the supernatant will be collected, and the recombinant lentivirus concentrated in DMEM without FBS. Primary $CD8^+$ T cells will next be transduced at multiplicity of infection (MOI) of ~5-10 in the presence of polybrene. Human recombinant IL-2 (R&D Systems) will be added every other day (50 IU/mL). T cells will be cultured for ~14 days after stimulation. Transduction efficiency of human primary T cells will be assessed by expression of a ZsGreen reporter gene (Clontech, Mountain View, CA).

E. Infusion of CAR T cells into a Patient

Prior to the *i.v.* infusion of anti-CD33 CAR T cells into the patient, cells will be washed with phosphate buffered saline and concentrated. A cell processor such as a Haemonetics CellSaver (Haemonetics Corporation, Braintree, MA), which provides a closed and sterile system, will be used for the washing and concentration steps before formulation.

The final T cells expressing the anti-CD33 chimeric receptors will be formulated into 100 ml of sterile normal saline supplemented with human serum albumin. Finally, patients will be infused with 1-10X10^7 T cells/kg over a period of 1-3 days (Maude et al., *NEJM* (2014) 371(16):1507). The number of T cells expressing anti-CD33 chimeric receptors infused will depend on numerous factors such as the state of the cancer patient, patient's age, prior treatment, etc.

Furthermore, also contemplated herein are immune cells expressing chimeric receptors that target CD45RA in addition to chimeric receptors that target CD33 in AML patients. This can be accomplished by two different approaches: 1) generating immune cells expressing anti-CD33 chimeric receptors and immune cells expressing anti-CD47RA chimeric receptors separately and infusing the patient with both types of immune cells separately, or 2) generating immune cells that target both CD33 and CD45RA simultaneously (Kakarla et al., *Cancer* (2):151 (2014)).

II. Autologous Hematopoietic Stem Cell Transplant (HSCT) Using $CD34^+CD33^-$ Cells

It is understood that the protocols regarding stem cell isolation from patients, conditioning regimens, as well as infusion of patients with stem cells vary greatly depending on the patient's age, condition, treatment history, and institution where the treatment is conducted. Thus, the protocol described below is merely an example and is subject to routine optimization by a person having ordinary skill in the art.

A. Isolation of Hematopoietic Stem Cells Using Peripheral Blood Stem Cell (PBSC) Mobilization Following Adoptive Transfer of anti-CD33 CAR T Cells

AML patient will be stimulated by i.v. administration of granulocyte colony-stimulating factor (G-CSF) 10 mg/kg per day. $CD34^+$ cell positive selection will be performed using immunomagnetic beads and an immunomagnetic enrichment device. A minimum of 2x 10^6 $CD34^+$ cells/kg body weight are expected to be collected using a Fenwall CS 3000+ cell separator (Park et al., *Bone Marrow Transplantation* (2003) 32:889).

B. Conditioning Regimen of a Patient

The conditioning regimen for autologous peripheral blood stem cell transplant (PBSCT) will be carried out using etoposide (VP-16) + cyclophosphamide (CY) + total body irradiation (TBI). Briefly, the regimen will consists of etoposide (VP-16) at 1.8g/m^2 i.v. constant infusion (c.i.v.) over 26 h as a single dose followed by cyclophosphamide (CY) at 60

mg/kg per day i.v. over 2 h for 3 days, followed by total body irradiation (TBI) at 300 cGy per day for the next 3 days.

To calculate the dose, ideal body weight or actual body weight, whichever is less, will be used. As previously mentioned, factors such as the state of the cancer patient, patient's age, prior treatment, as well as the type of institution where the procedure is conducted will all be taken into consideration when determining the precise conditioning regimen.

C. Plasmid Construction of CRISPR-Cas9 System targeting CD33

The lentiCRISPR v2 containing inserts Cas9 and Puromycin resistance will be obtained from Addgene (Plasmid #52961) (Sanjana et al., *Nat Methods* (2014) (8):783). To clone the single guide RNA (sgRNA) CD33 guide sequence, the lentiCRISPR v2 will be cut and dephosporylated with FastDigest BsmBI and FastAP (Fermentas) at 37 °C for 2 hours. gRNA targeting CD33 will be designed using the online optimized design tool at crispr.mit.edu. Alternatively, gRNA will have a sequence depicted in FIGURE 12 (SEQ ID NO:11). CD33 gRNA oligonucleotides will be obtained from Integrated DNA Technologies (IDT), phosphorylated using polynucleotide kinase (Fermentas) at 37 °C for 30 minutes and annealed by heating to 95 °C for 5 minutes and cooling to 25 °C at 1.5 °C/minute. T7 ligase will be used to anneal the oligos, after which the annealed oligos will be ligated into gel purified vector (Qiagen) at 25 °C for 5 minutes. Resulting plasmid can then be amplified using an endotoxin-free midi-prep kit (Qiagen) (Sanjana et al., *Nat Methods* (2014)(8):783).

Alternatively, a two vector system may be used (where gRNA and Cas are expressed from separate vectors) protocol described previously (Mandal et al., *Cell Stem Cell* (2014) 15(5):643). Here, Mandal et al. achieved efficient ablation of genes in human hematopoietic stem cells using CRISPR-Cas system expressed from non-viral vectors.

Briefly, human-codon-optimized Cas9 gene containing a C-terminal SV40 nuclear localization signal will be cloned into a CAG expression plasmid with 2A-GFP. To direct Cas9 to cleave CD33 sequences of interest, the guide RNA (gRNA (SEQ ID. NO. 11)) will be separately expressed from a plasmid containing the human U6 polymerase III promoter. gRNA sequence oligonucleotides will be obtained from Integrated DNA Technologies (IDT), annealed, and introduced into the plasmid using BbsI restriction sites. Due to the transcription initiation requirement of a 'G' base for human U6 promoter, as well as the requirement for the PAM (protospacer-adjacent motif) sequence, genome target will comprise $GN_{20}GG$ nucleotide sequence.

In addition to infusing patients with CD33 depleted hematopoietic stem cells HSCs, a protocol will be developed in which the patients are subsequently infused with CD45RA depleted HSCs. Alternatively, the inventors will generate $CD34^+CD33^-CD45RA^-$ cells using CRISPR-Cas9 system to reduce both CD33 and CD45RA genes simultaneously. Example guide RNA sequences for CD45RA and CD33 are shown in Tables 4 and 5).

D. Transfection of $CD34^+$ cells HSCs to generate $CD34^+CD33^-$ cells

Freshly isolated peripheral blood-derived $CD34^+$ cells (from step 4) will be seeded at 1×10^6 cells/ml in serum-free CellGro SCGM Medium in the presence of cells culture grade Stem Cell Factor (SCF) 300 ng/ml, FLT3-L 300 ng/ml, Thrombopoietin (TPO) 100 ng/ml and IL-3 60 ng/ml. Following 24 hour of pre-stimulation, $CD34^+$ HSCs will be transfected with LentiCRISPR v2 containing Cas9 and CD33 gRNA using Amaxa Human CD34 cell Nucleofector kit (U-008) (#VPA-1003) (Mandal et al., *Cell Stem Cell* (2014) 15(5):643). 24-48 hours post-transfection, $CD34^+$ $CD33^+$ cells are selected with 1.2 μg/ml puromycin. Following the puromycin selection, $CD34^+CD33^-$ cells will be maintained in puromycin-free media for couple of days.

E. Reinfusion of $CD34^+CD33^-$ cells into the patient

$CD34^+$ cells transfected *ex vivo* with CRISPR-Cas9-CD33 ($CD34^+CD33^-$ cells) are immediately reinfused through a Hickman catheter using a standard blood administration set without a filter (Hacein-Bey Abina et al. *JAMA* (2015) 313(15):1550.

Generally, patients who have undergone the above outlined treatment protocol will be monitored for the reappearance of circulating blasts and cytopenias. Additionally, depending on the underlying mechanism of AML in a specific patient, the success of the treatment will be monitored by testing for reappearance of an informative molecular or cytogenetic marker, or an informative flow cytometry pattern. For example, reemergence of a BCR-ABL signal in Philadelphia chromosome–positive AML will be detected using fluorescent in situ hybridization (FISH) with probes for BCR (on chromosome 22) and ABL (on chromosome 9).

To evaluate the success of CD33 deletion via CRISPR-Cas9 system, peripheral blood $CD34^+$ cells will be isolated from patients (post-transplant) and assessed for the CD33 expression, for example using flow cytometry, Western blotting, or immunohistochemistry.

As described herein, the HSCT described in this Example can be either autologous or allogeneic, and both approaches are suitable and can be incorporated in the methods described in the present disclosure.

III. Optional Step: Continued treatment of a patient with a CD33 antibody attached to a toxin

A. Treatment of Patients with CD33 Immunotoxin Gemtuzumab Ozogamicin (GO)

Patients will be treated with 9 mg/m^2 of anti-CD33 antibody gemtuzumab ozogamicin (GO) as a 2-hour intravenous infusion in 2 doses separated by 2 weeks (Larson et al., *Cancer* (2005), 104(7):1442-52). GO is comprised of a humanized monoclonal antibody against CD33 which is conjugated with a cytostatic agent, calicheamicin (FIGURE 8).

Alternatively, the anti-CD33 antibodies may be conjugated to different toxins, such as diphtheria toxin, Pseudomonas exotoxin A (PE), or ricin toxin A chain (RTA) can be generated (Wayne et al., *Blood* (2014) 123(16): 2470). Similarly, anti-CD45RA antibodies may be attached to a toxin and included in the treatment regimen.

EXAMPLE 4: T cells and NK cell lines expressing an anti-CD33 chimeric receptor induce cell death of target cells expressing CD33

Binding of chimeric receptors to CD33

Chimeric receptors that bind CD33 (*e.g.*, CART1, CART2, CART3) were generated using convention recombinant DNA technologies and inserted into a pHIV-Zsgreen vector (Addgene; Cambridge, MA). The vectors containing the chimeric receptors were used to generate lentiviral particles, which were used to transduce different cell types, for example T cell lines (*e.g.*, 293 T cells) and NK cell lines (*e.g.*, NK92 cells). Expression of the chimeric receptors was detected by Western blotting (FIGURE 9, panel A) and flow cytometry (FIGURE 9, panel B).

Cells expressing the chimeric receptors were selected by fluorescence-activated cell sorting (FACS) and assessed for their ability to bind CD33. Briefly, lysates of 293T cells expressing the chimeric receptors were coincubated with CD33 or CD33-allophycocyanin (APC) conjugate. The samples were subjected to protein electrophoresis and either stained with Ponceau protein stain (FIGURE 10, panel A) or transferred to a membrane and probed with an anti-CD3ζ primary antibody (FIGURE 10, panel B). In both cases, binding between the chimeric receptors and their target, CD33.

K562 cells expressing the chimeric receptors were also assessed for binding to CD33 by flow cytometry using CD33 as a probe (FIGURE 10, panel C). There was an increase in the number of cells positive for expression of the chimeric receptor (CART1, CART2, or

CART3) and CD33 binding as compared to cells containing an empty vector control, indicating the chimeric receptors bind to CD33.

Cytotoxicity induced by cells expressing the chimeric receptors

NK-92 cells expressing the chimeric receptors were functionally characterized for the ability to induce cytotoxicity of target cells presenting CD33 on the cell surface (*e.g.*, K562 are a human chronic myelogenous leukemia cell line that are CD33+). To perform the cytotoxicity assays, effector cells (immune cells, such as NK-92 cells) were infected with lentivirus particles encoding the chimeric receptors and expanded. Seven days post infection, cells expressing the chimeric receptors were selected by FACS analysis by selecting for fluorescent markers also encoded by the chimeric receptor encoding vector (*e.g.*, GFP+ or Red+). The selected cells that express the chimeric receptors were expanded for one week. Fourteen days post infection, the cytotoxicity assay was performing involving staining the target cells (cells expressing the target cell-surface lineage-specific antigen, CD33) with carboyxfluorescein succinimidyl ester (CFSE) and counting both the target cells and cells expressing the chimeric receptors. Different ratios of target cells and cells expressing the chimeric receptors were coincubated in round bottom 96-well plates for 4.5 hrs, after which 7-aminoactinomycin D (7-AAD) was added to stain non-viable cells. Flow cytometry was performed to enumerate the population of viable and non-viable target cells. As shown in FIGURE 11, panels A and B, NK92 cells expressing chimeric receptors CART1, CART2, or CART3 induced a substantial amount of cell death of target K562 cells at each of the cell ratios assessed.

To determine that the cell death of K562 cells was dependent on specific targeting of the chimeric receptor to CD33, K562 were genetically engineered to be deficient in CD33 using a CRISPR/Cas system. Briefly, a human codon-optimized Cas9 endonuclease and a gRNA targeting a portion of the IgC domain of CD33 were expressed in the K562 cells, resulting in populations of CD33-deficient K562 cells. The cells were expanded and co-incubated with NK92 cells expressing the chimeric receptors, and the cytotoxicity assay was performed as described above. As shown in FIGURE 12 panel A, the pooled CD33-deficient K562 cells showed a modest reduction in cell death with co-incubated with the NK92 cells expressing the chimeric receptors. However, when single clones of CD33-deficient K562 cells were isolated, expanded, and used to perform the cytotoxicity assays, a more significant reduction in cytotoxicity was observed (FIGURE 12, panel B).

Expression of chimeric receptors in primary T cells

Primary T cell populations were isolated from PMBCs obtained from donors by FACS by positively selecting CD4+, CD8+, or CD4+/CD8+ cells, resulting in highly pure populations (FIGURE 13, panels A and B). Each of the populations of primary T cells (CD4+, CD8+, or CD4+/CD8+ cells) were transduced with a lentiviral vector containing the chimeric receptors (*e.g.*, CART1 and CART8) and the resulting primary T cells expressing chimeric receptors were used to perform cytotoxicity assays, as described above. Co-incubation of the population of CD4+ T cells expressing the chimeric receptors with K562 (1000 target K562 cells) did not result in cytotoxicity of the K562 cells (FIGURE 14, panel A). In contrast, in a cytotoxicity assay using either CD8+ or CD4+/CD8+ cells expressing the chimeric receptors and 1000 target K562 cells, the CD8+ or CD4+/CD8+ cells were able to induce cell death of the K562 cells at a low cellular ratio (FIGURE 14, panel B).

Genetic engineering human hematopoietic stem cells

Several gRNAs were designed to hybridize to the IgC domain of CD33 (see, for example, Table 4, SEQ ID NO: 11 or 28-31). Each of the gRNAs were expressed along with a Cas9 endonculease in K562 cells. The expression of CD33 was assessed by flow cytometry (FIGURE 15). As shown for Crispr 3 (SEQ ID NO: 28) and Crispr5 (SEQ ID NO: 29), a significant reduction in CD33 was found in cells expressing the CD33-targeting CRISPR/Cas system, as compared to control cells expressing CD33.

The CD33-deficient hematopoietic stem cells were also assessed for various characteristics, including proliferation, erythopoeitic differentiation, and colony formation. Briefly, CD33-deficient hematopoietic stem cells and control cells were induced to differentiate by exposing the cells to hemin, and CD71, a marker of erythroid precursors, was assessed by flow cytometry at different time points (FIGURE 16, panels A and B). CD33-deficient hematopoietic stem cells underwent erythopoeitic differentiation and flow cytometric profiles appeared similar to the control cells (CD33+). The cells were also subjected to MTT assay to measure the metabolic activity of the CD33-deficient hematopoietic stem cells. As shown in FIGURE 16, panel C, the CD33-deficient hematopoietic stem cells performed comparably to the control cells. Finally, the ability of the cells to proliferate and form colonies of cells was observed using a microscopic colony formation assay. Again, the CD33-deficient hematopoietic stem cells were able to form colonies to a similar extents as the control cells (FIGURE 18). These results indicate the

CRISPR/Cas deletion of a portion of CD33 does not significantly impact the ability of the cells to proliferate, differentiate, or form colonies.

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one of skill in the art can easily ascertain the essential characteristics of the present disclosure, and without departing from the spirit and scope thereof, can make various changes and modifications of the disclosure to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e. "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of." "Consisting essentially of," when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements

and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

WHAT IS CLAIMED IS:

1. A method of treating a hematopoietic malignancy, comprising administering to a subject in need thereof:

(i) an effective amount of an agent targeting a lineage-specific cell-surface antigen, wherein the agent comprises an antigen-binding fragment that binds the lineage-specific cell-surface antigen; and

(ii) a population of hematopoietic cells that are deficient in the lineage-specific cell-surface antigen.

2 The method of claim 1, wherein the agent is an immune cell expressing a chimeric receptor that comprises the antigen-binding fragment that binds the lineage-specific cell-surface antigen.

3. The method of claim 1 or claim 2, wherein the immune cells, the hematopoietic cells, or both, are allogeneic or autologous.

4 The method of any one of claims 1-3, wherein the hematopoietic cells are hematopoietic stem cells.

5. The method of any one of claims 1-4, wherein the lineage-specific cell-surface antigen is a type 2 lineage-specific cell-surface antigen.

6. The method of claim 5, wherein the type 2 lineage-specific cell-surface antigen is CD33.

7. The method of any one of claims 1-6, wherein the antigen-binding fragment in the chimeric receptor is a single-chain antibody fragment (scFv) that specifically binds the lineage-specific cell-surface antigen, which is a human protein.

8. The method of claim 7, wherein the scFv binds to human CD33.

9. The method of claim 8, wherein the scFv comprises a heavy chain variable region, which has the same complementary determining regions (CDRs) as those in SEQ ID

NO:12, and a light chain variable region, which has the same CDRs as those in SEQ ID NO:13.

10. The method of claim 9, wherein the scFv comprises a heavy chain variable domain having the amino acid sequence of SEQ ID NO: 12 and a light chain variable domain having the amino acid sequence of SEQ ID NO: 13.

11. The method of any one of claims 1-4, wherein the lineage-specific cell-surface antigen is a type 1 lineage-specific cell-surface antigen.

12. The method of claim 11, wherein the type 1 lineage-specific cell-surface antigen is CD19.

13. The method of any one of claims 1-12, wherein the immune cells are T cells.

14. The method of any one of claims 1-13, wherein the chimeric receptor further comprises:

(a) a hinge domain
(b) a transmembrane domain,
(c) at least one co-stimulatory domain,
(d) a cytoplasmic signaling domain, or
(e) a combination thereof.

15. The method of claim 14, wherein the chimeric receptor comprises at least one co-stimulatory signaling domain, which is derived from a co-stimulatory receptor selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen- 1 (LFA- 1), CD2, CD7, LIGHT, NKG2C, B7-H3, GITR, HVEM, and a combination thereof.

16. The method of claim 15, wherein the at least one co-stimulatory signaling domain is derived from 4-1BB, CD28, or ICOS.

17. The method of claim 16, wherein the at least one co-stimulatory signaling domain comprises a signaling domain of CD28 and a signaling domain of ICOS.

18. The method of claim 16 or claim 17, wherein the at least one co-stimulatory signaling domain is from CD28 and the chimeric receptor further comprises a second co-stimulatory signaling domain from 4-1BB or ICOS.

19. The method of any one of claims 1-18, wherein the chimeric receptor comprises a cytoplasmic signaling domain, which is from CD3ζ.

20. The method of any one of claims 1-19, wherein the chimeric receptor comprises a hinge domain, which is from CD8α or CD28α.

21. The method of any one of claims 1-20, wherein the chimeric receptor comprises a transmembrane domain, which is from CD8, CD28, or ICOS.

22. The method of any one of claims 1-21, wherein the chimeric receptor comprises, from N terminus to C terminus,

(i) a scFv that binds to the lineage-specific cell-surface antigen, a hinge domain from CD8α, a transmembrane domain from CD8, a costimulatory domain from 4-1BB, and a cytoplasmic signaling domain from CD3ζ;

(ii) a scFv that binds to the lineage-specific cell-surface antigen, a hinge domain from CD8α, a transmembrane domain from CD28, a costimulatory domain from CD28, and a cytoplasmic signaling domain from CD3ζ; or

(iii) a scFv that binds to the lineage-specific cell-surface antigen, a hinge domain from CD8α, a transmembrane domain from CD28, a first costimulatory domain from CD28, a second costimulatory domain from 4-1BB, and a cytoplasmic signaling domain from CD3ζ.

23. The method of any one of claims 4-22, wherein the hematopoietic stem cells are $CD34^{+}/CD33^{-}$.

24. The method of claim 23, wherein the hematopoietic stem cells are from bone marrow cells or peripheral blood mononuclear cells (PBMCs).

25. The method of any one of claims 1-24, wherein the subject has Hodgkin's lymphoma, non-Hodgkin's lymphoma, leukemia, or multiple myeloma.

26. The method of claim 25, wherein the subject has leukemia, which is acute myeloid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, or chronic lymphoblastic leukemia.

27. A nucleic acid comprising a nucleotide sequence encoding a chimeric receptor, wherein the chimeric receptor comprises an antigen-binding fragment that binds CD33, a transmembrane domain, and a cytoplasmic signaling domain,

wherein the antigen-binding fragment comprises a heavy chain variable region, which has the same CDRs as those in SEQ ID NO: 12, and a light chain variable region, which has the same CDRs as those in SEQ ID NO: 13.

28. The nucleic acid of claim 27, wherein the chimeric receptor further comprises at least one costimulatory domain.

29. The nucleic acid of claim 28, wherein the at least one co-stimulatory signaling domain is derived from a co-stimulatory receptor selected from the group consisting of CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen- 1 (LFA- 1), CD2, CD7, LIGHT, NKG2C, B7-H3, GITR, HVEM, and a combination thereof.

30. The nucleic acid of claim 29, wherein the at least one co-stimulatory signaling domain is derived from 4-1BB, CD28, or ICOS.

31. The nucleic acid of claim 30, wherein the at least one co-stimulatory signaling domain comprises a signaling domain of CD28 and a signaling domain of ICOS.

332. The nucleic acid of claim 30 or claim 31, wherein the at least one co-stimulatory signaling domain is from CD28 and the chimeric receptor further comprises a second co-stimulatory signaling domain from 4-1BB or ICOS.

33. The nucleic acid of any one of claims 27-32, wherein the cytoplasmic signaling domain is from CD3ζ.

34. The nucleic acid of any one of claims 27-33, wherein the hinge domain is from CD8α or CD28α.

35. The nucleic acid of any one of claims 27-34, wherein transmembrane domain is from CD8, CD28, or ICOS.

36. The nucleic acid of any one of claims 27-35, wherein the chimeric receptor comprises, from N terminus to C terminus,

(i) the antigen-binding fragment, a hinge domain from CD8α, a transmembrane domain from CD8, a costimulatory domain from 4-1BB, and a cytoplasmic signaling domain from CD3ζ;

(ii) the antigen-binding fragment, a hinge domain from CD8α, a transmembrane domain from CD28, a costimulatory domain from CD28, and a cytoplasmic signaling domain from CD3ζ; or

(iii) the antigen-binding fragment, a hinge domain from CD8α, a transmembrane domain from CD28, a first costimulatory domain from CD28, a second costimulatory domain from 4-1BB, and a cytoplasmic signaling domain from CD3ζ.

37. The nucleic acid of any one of claims 27-36, wherein the antigen-binding fragment comprises

a heavy chain variable domain having the amino acid sequence of SEQ ID NO: 12 and

a light chain variable domain having the amino acid sequence of SEQ ID NO: 13.

38. A vector comprising the nucleic acid of any one of claims 27-37.

39. A chimeric receptor encoded by the nucleic acid of any one of claims 27-38.

40. An immune cell expressing the chimeric receptor of claim 39.

41. The immune cell of claim 40, which is a T cell.

42. The immune cell of claim 40 or claim 41, wherein the immune cell is obtained from a patient having a hematopoietic malignancy.

43. A genetically engineered hematopoietic cell that is deficient in a lineage-specific cell-surface antigen, which presents on the hematopoietic cell before genetic engineering.

44. The genetically engineered hematopoietic cell of claim 43, wherein the whole or a portion of an endogenous gene encoding the lineage-specific cell-surface antigen is deleted.

45. The genetically engineered hematopoietic cell of claim 44, wherein the whole or portion of an endogenous gene encoding the lineage-specific cell-surface antigen is deleted using genome editing.

46. The genetically engineered hematopoietic cell of claim 45, wherein the genome editing involves a zinc finger nuclease (ZFN), a transcription activator-like effector-based nuclease (TALEN), or a CRISPR-Cas system.

47. The genetically engineered hematopoietic cell of any one of claims 43-46, wherein the lineage-specific cell-surface antigen is CD33 or CD19.

48. The genetically engineered hematopoietic cell of claim 46, wherein the lineage-specific cell-surface antigen is CD33 and a portion of the immunoglobulin constant (IgC) domain of the CD33 is deleted.

49. The genetically engineered hematopoietic cell of any one of claims 43-48, wherein the hematopoietic cell is a hematopoietic stem cell.

50. The genetically engineered hematopoietic stem cell of claim 49, which is a $CD34^{+}/CD33^{-}$ cell.

51. The genetically engineered hematopoietic cell of any one of claims 43-50, which are from bone marrow cells or peripheral blood mononuclear cells (PBMCs).

52. A method of producing a cell that is deficient in a lineage-specific cell-surface antigen, comprising:

providing a cell, and

introducing into the cell

(i) a nucleic acid that comprises a nucleotide sequence of a CRISPR-Cas system guide RNA (gRNA), which hybridizes to a portion of the nucleotide sequence that encodes the lineage-specific cell-surface antigen, and

(ii) a Cas endonuclease.

53. The method of claim 52, wherein the Cas endonuclease is Cas9 or Cpf1.

54. The method of claim 52 or 53, wherein (i) and (ii) are encoded on the same nucleic acid or on different nucleic acids.

55. The method of any of claims 52-54, wherein (i) and (ii) are introduced into the cell as a pre-formed ribonucleoprotein complex.

56. The method of any one of claims 52-55, wherein the portion of the nucleotide sequence to which the gRNA hybridizes consists of 18-22 nucleotides.

57. The method of any one of claims 52-56, wherein the lineage-specific cell-surface antigen is CD33 or CD19.

58. The method of any one of claims 52-57, wherein the cell is a hematopoietic cell.

59. The method of claim 58, wherein the hematopoietic cell is a hematopoietic stem cell.

60. The method of claim 59, wherein the hematopoietic stem cell is $CD34^+$.

61. The method of any one of claims 52-60, wherein the gRNA comprises the nucleotide sequence of any one of SEQ ID NO: 11 and 28-31.

62. A kit comprising:

(i) an immune cell of any one of claims 40-42 and an agent that targets a lineage-specific, cell-surface antigen, which comprises an antigen binding fragment that binds the lineage-specific cell-surface antigen; and

(ii) a population of hematopoietic cells that are deficient in the lineage-specific cell-surface antigen as set forth in any one of claims 43-51.

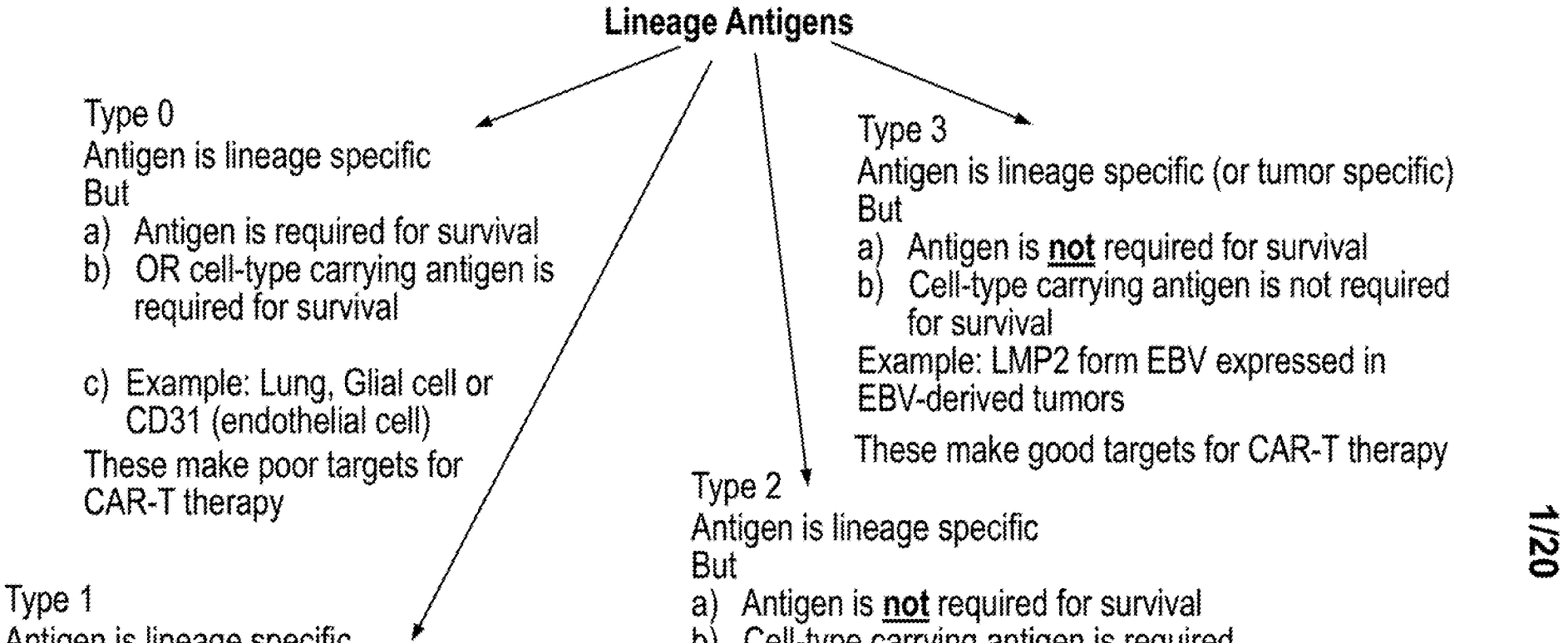

Lineage Antigens
Type 0
Antigen is lineage specific
But
a) Antigen is required for survival
b) OR cell-type carrying antigen is required for survival
c) Example: Lung, Glial cell or CD31 (endothelial cell)
These make poor targets for CAR-T therapy
Type 1
Antigen is lineage specific
But
a) Antigen is not required for survival
b) Cell-type carrying antigen is not required for survival
Example: CD307 expressed in Myeloma cells and Plasma Cells
These make good targets for CAR-T therapy
Type 2
Antigen is lineage specific
But
a) Antigen is not required for survival
b) Cell-type carrying antigen is required for survival
c) But ANTIGEN CAN BE CRISPED in NORMAL CELL OR STEM CELL
Example: CD33 expressed in AML and Myeloid cells and stem cells
These make good targets for CAR-T therapy if they can be combined with CRISPR-mediated deletion of antigen in normal cells
Type 3
Antigen is lineage specific (or tumor specific)
But
a) Antigen is not required for survival
b) Cell-type carrying antigen is not required for survival
Example: LMP2 form EBV expressed in EBV-derived tumors
These make good targets for CAR-T therapy

FIG. 1

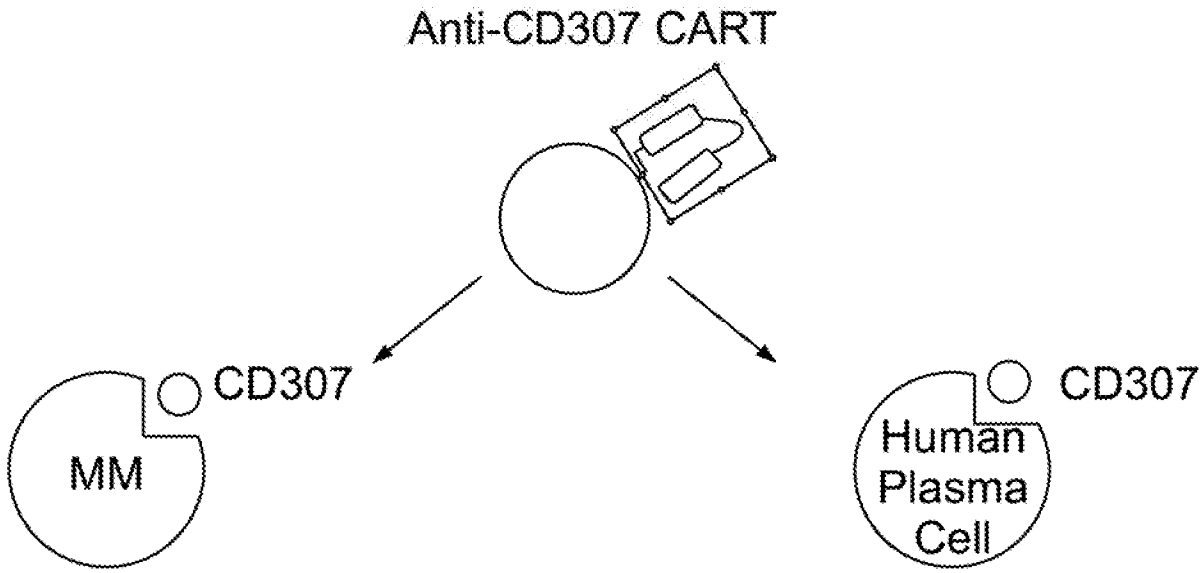

Anti-CD307 CART
CD307
MM
CD307
Human
Plasma
Cell

FIG. 2

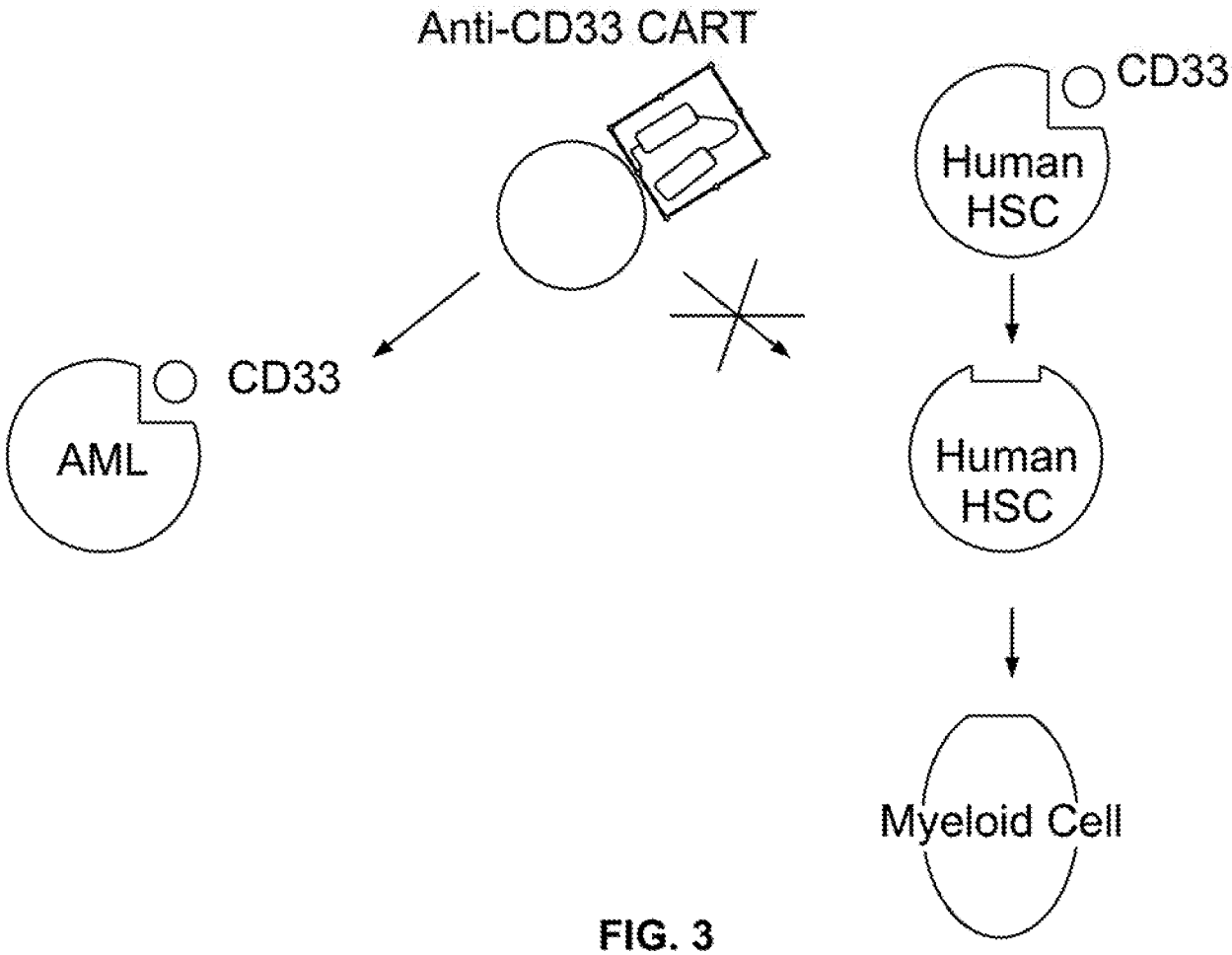

Anti-CD33 CART
CD33
Human
HSC
CD33
AML
Human
HSC
Myeloid Cell

FIG. 3

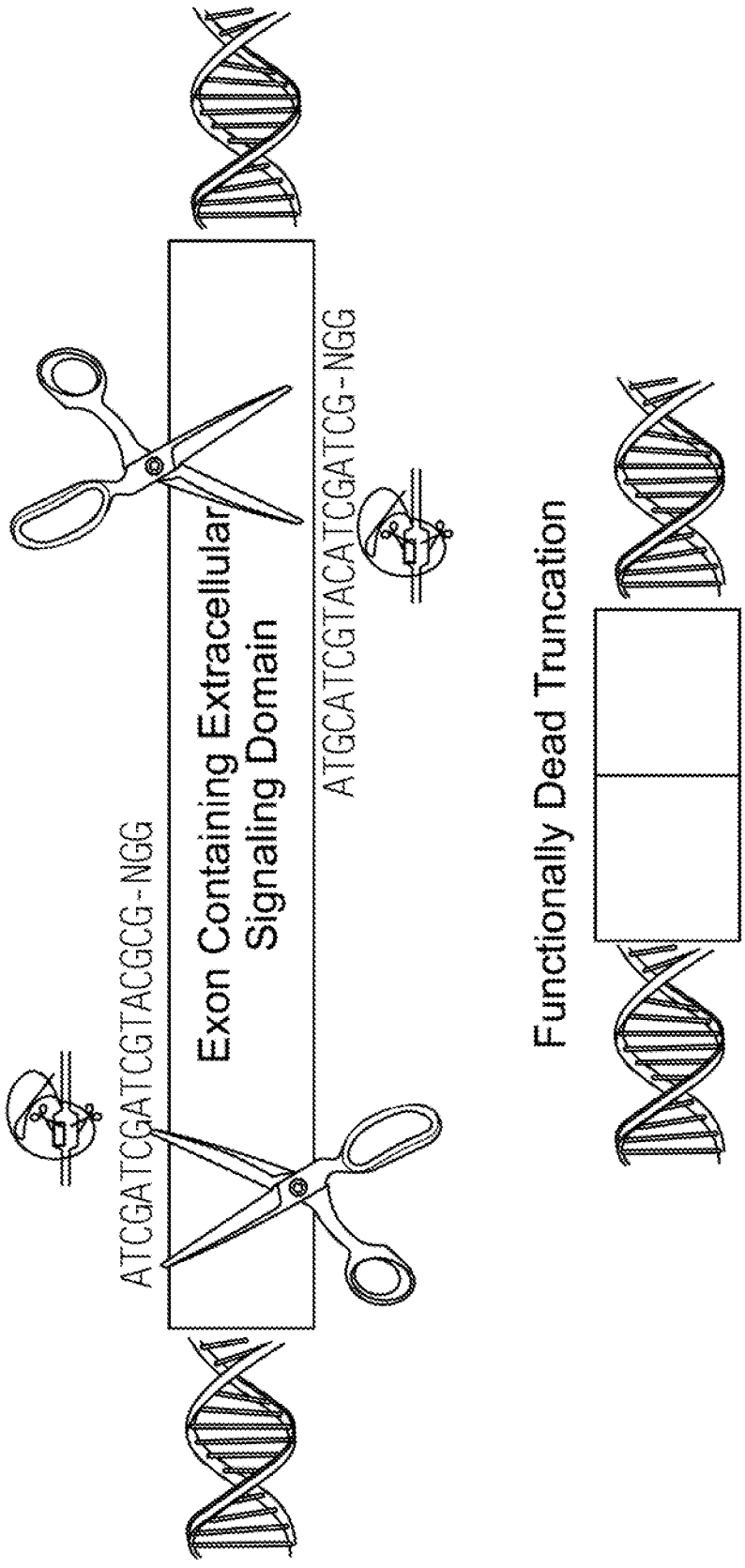

ATCGATCGATCGTACGGCG-NGG
Exon Containing Extracellular Signaling Domain
ATGCATCGTACATCGATCG-NGG
Functionally Dead Truncation

FIG. 4

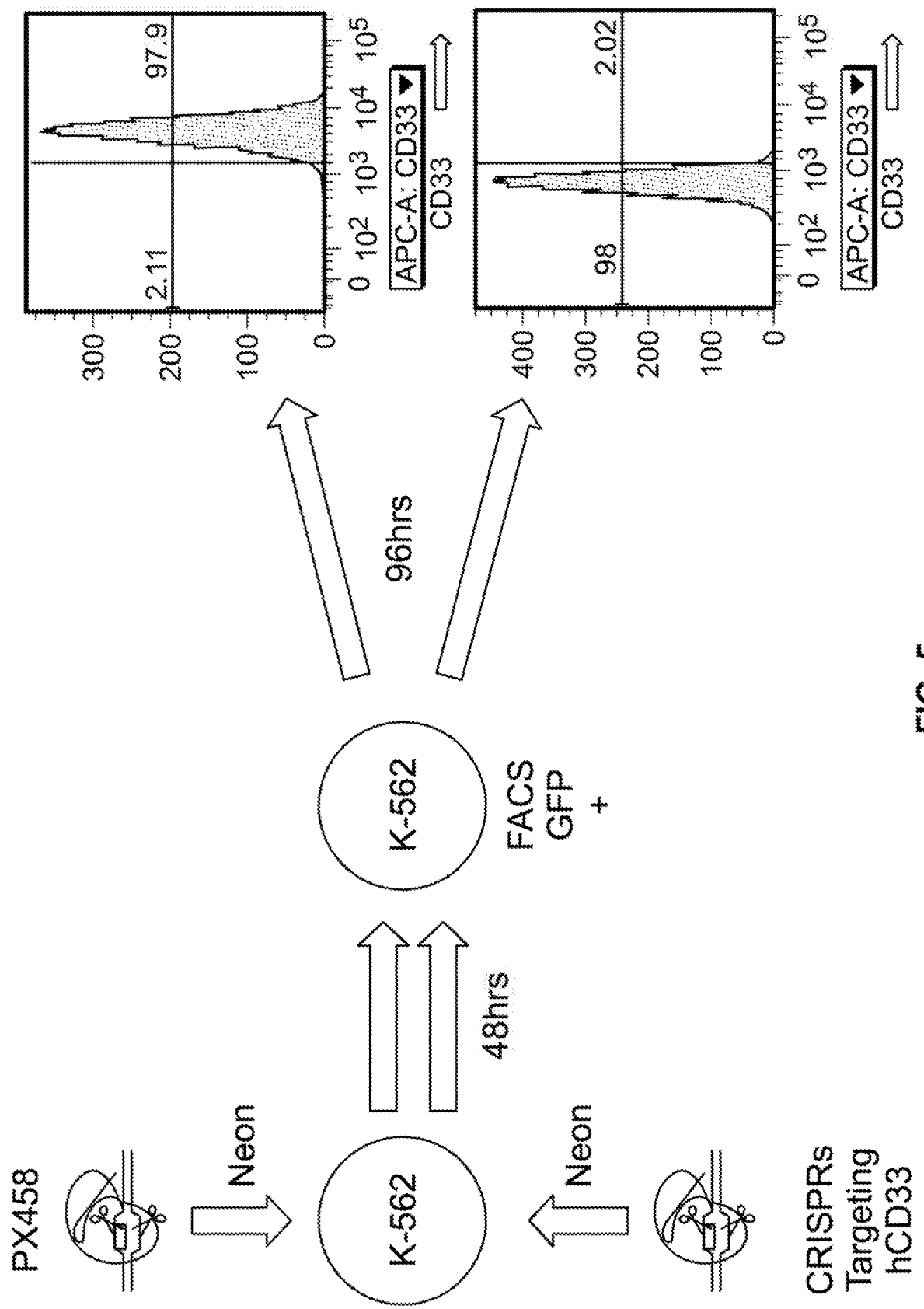

PX458
Neon
K-562
48hrs
Neon
CRISPRs Targeting hCD33
K-562
FACS GFP +
96hrs
97.9
2.11
APC-A: CD33
CD33
2.02
98
APC-A: CD33
CD33

FIG. 5

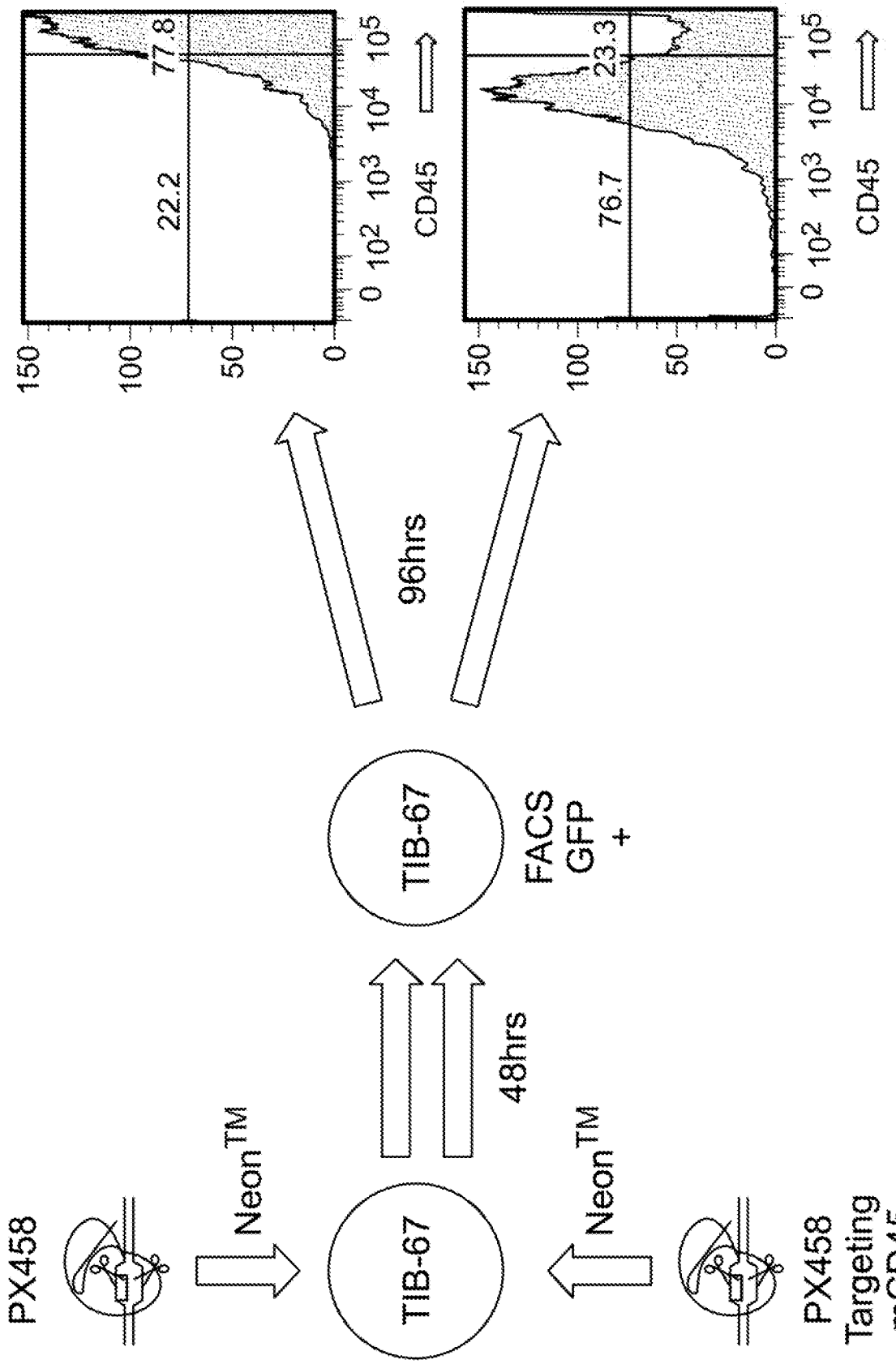

PX458
Neon™
TIB-67
48hrs
Neon™
PX458
Targeting
mCD45
exon8
TIB-67
FACS
GFP
+
96hrs
77.8
22.2
CD45
23.3
76.7
CD45

FIG. 6

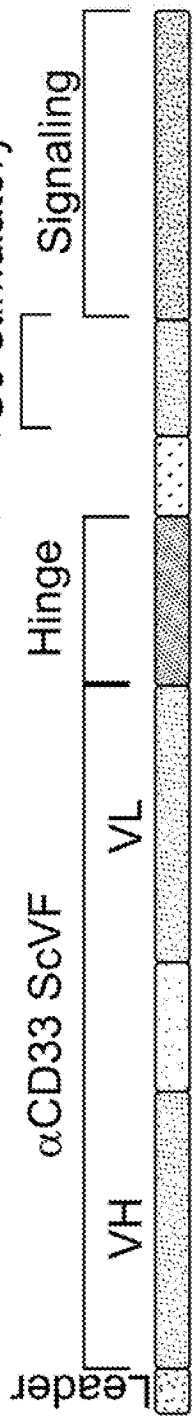


FIG. 7A

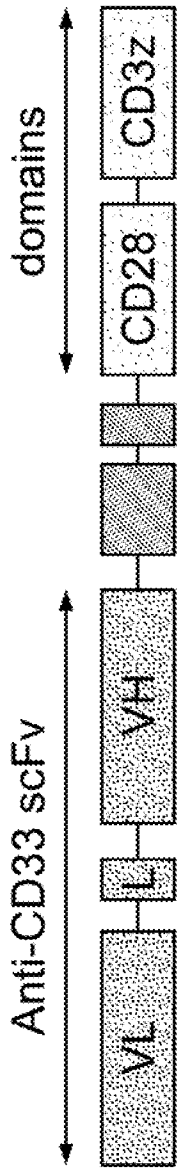


FIG. 7B

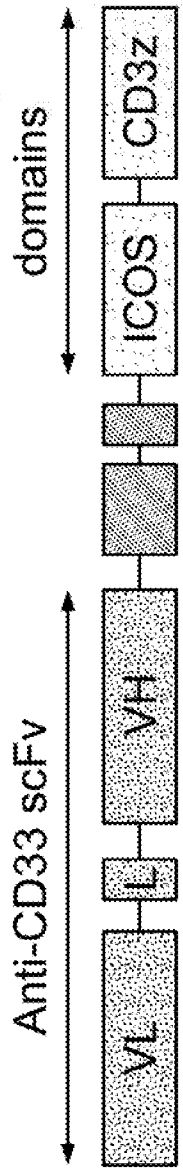


FIG. 7C

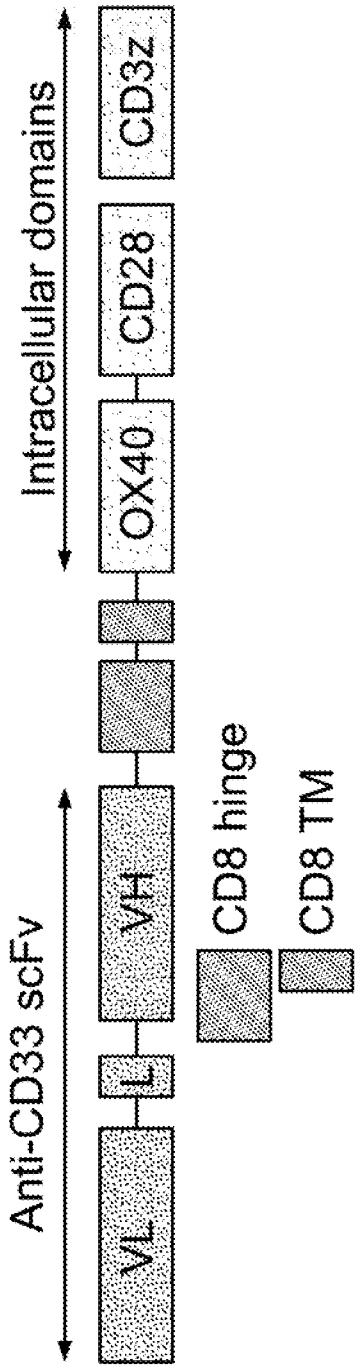

Anti-CD33 scFv
VL
L
VH
CD8 hinge
CD8 TM
Intracellular domains
OX40
CD28
CD3z

FIG. 7D

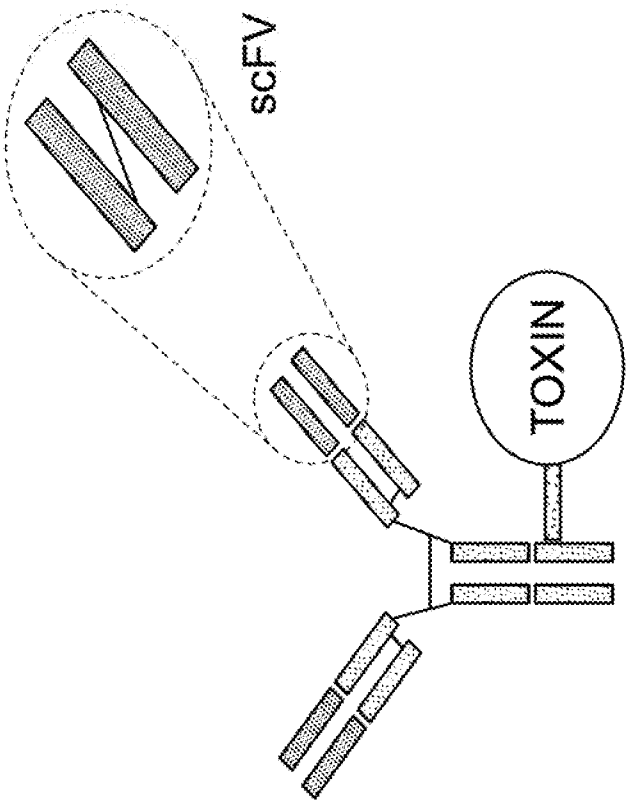
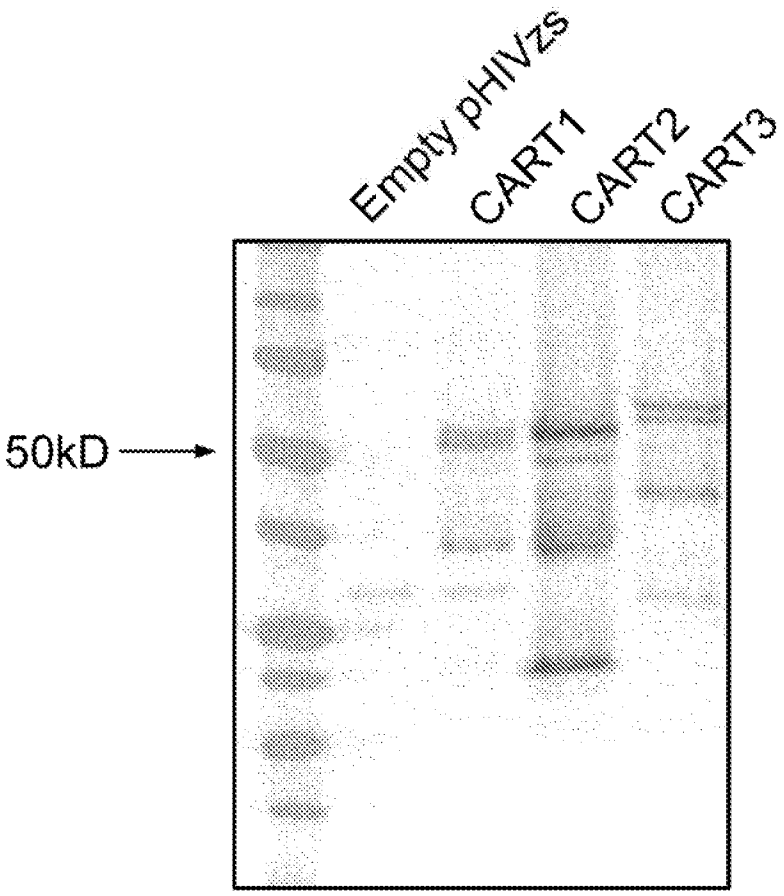

scFV
TOXIN
mAb

FIG. 8



Stain with anti-CD3TCRz, ab
Epitope: C-term CD3z

Construct	MW kDa
CART1	55.3AAAA
CART2	56.3
CART3	61

FIG. 9A

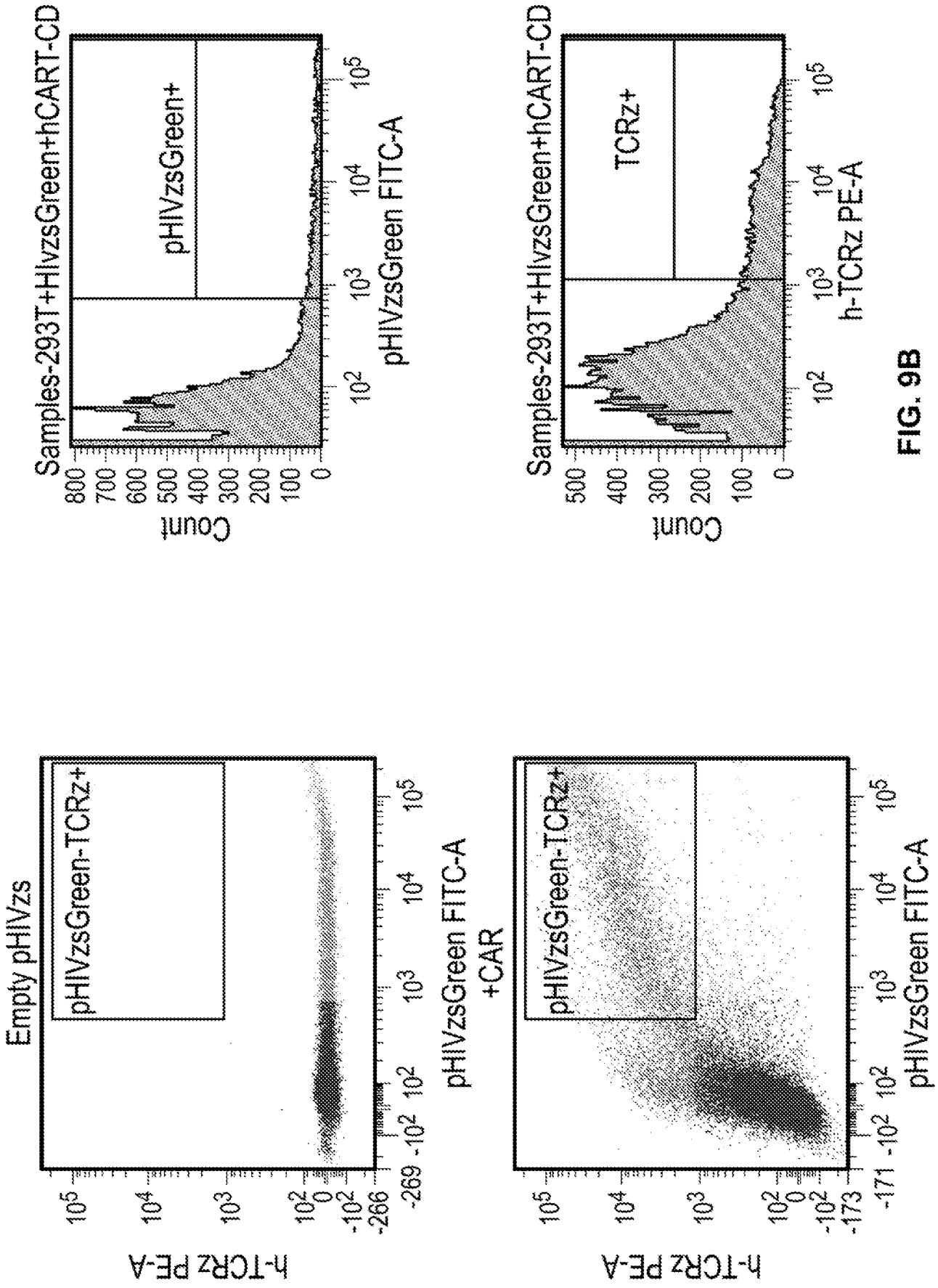

Samples-293T+HIvzsGreen+hCART-CD
pHIVzsGreen+
Count
pHIVzsGreen FITC-A
Samples-293T+HIvzsGreen+hCART-CD
TCRz+
Count
h-TCRz PE-A
Empty pHIVzs
pHIVzsGreen-TCRz+
h-TCRz PE-A
pHIVzsGreen FITC-A
+CAR
pHIVzsGreen-TCRz+
h-TCRz PE-A
pHIVzsGreen FITC-A

FIG. 9B

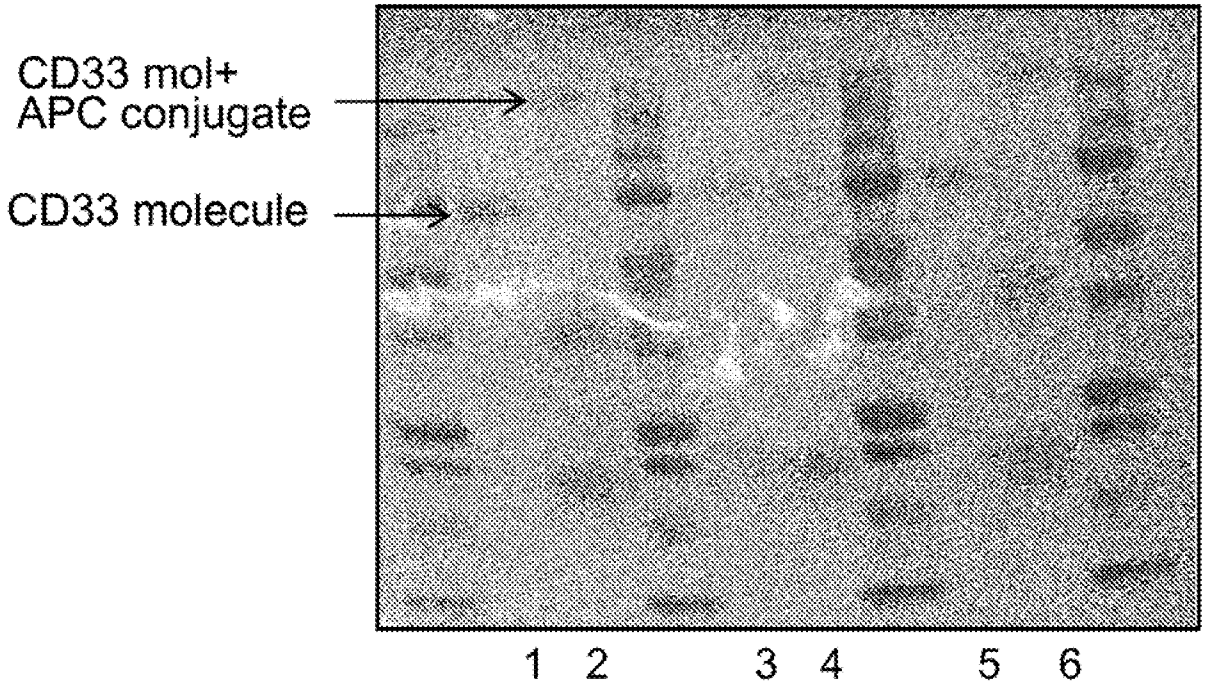


FIG. 10A

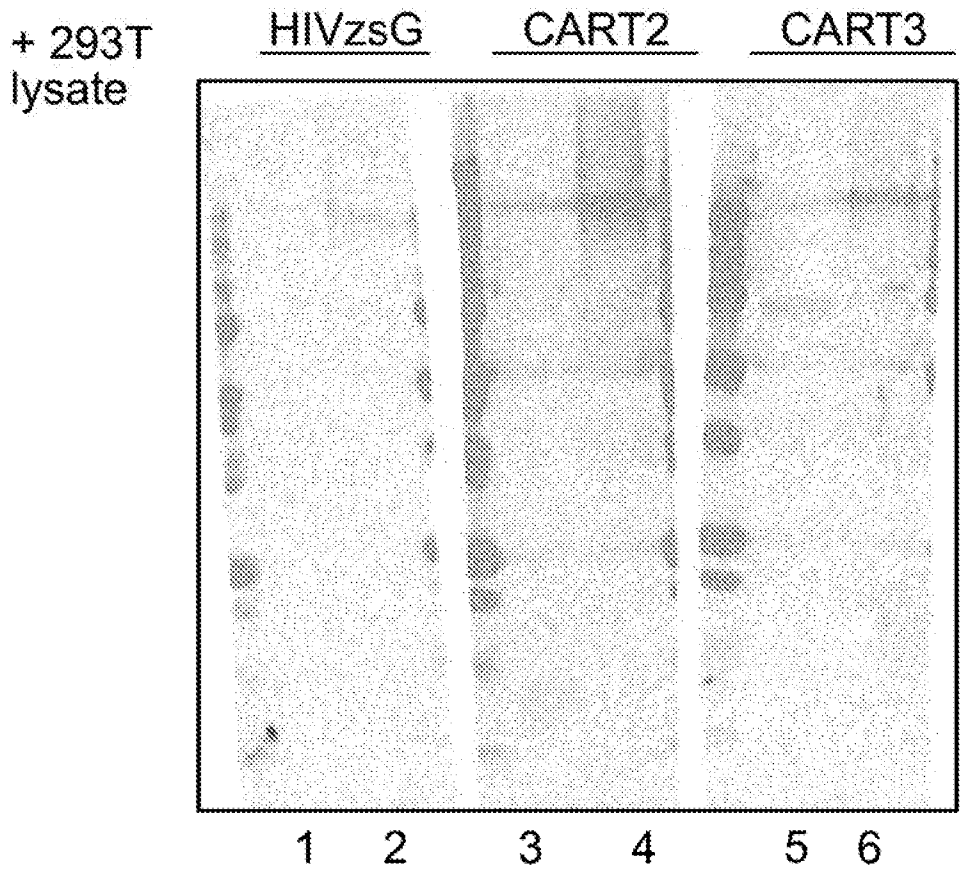


FIG. 10B

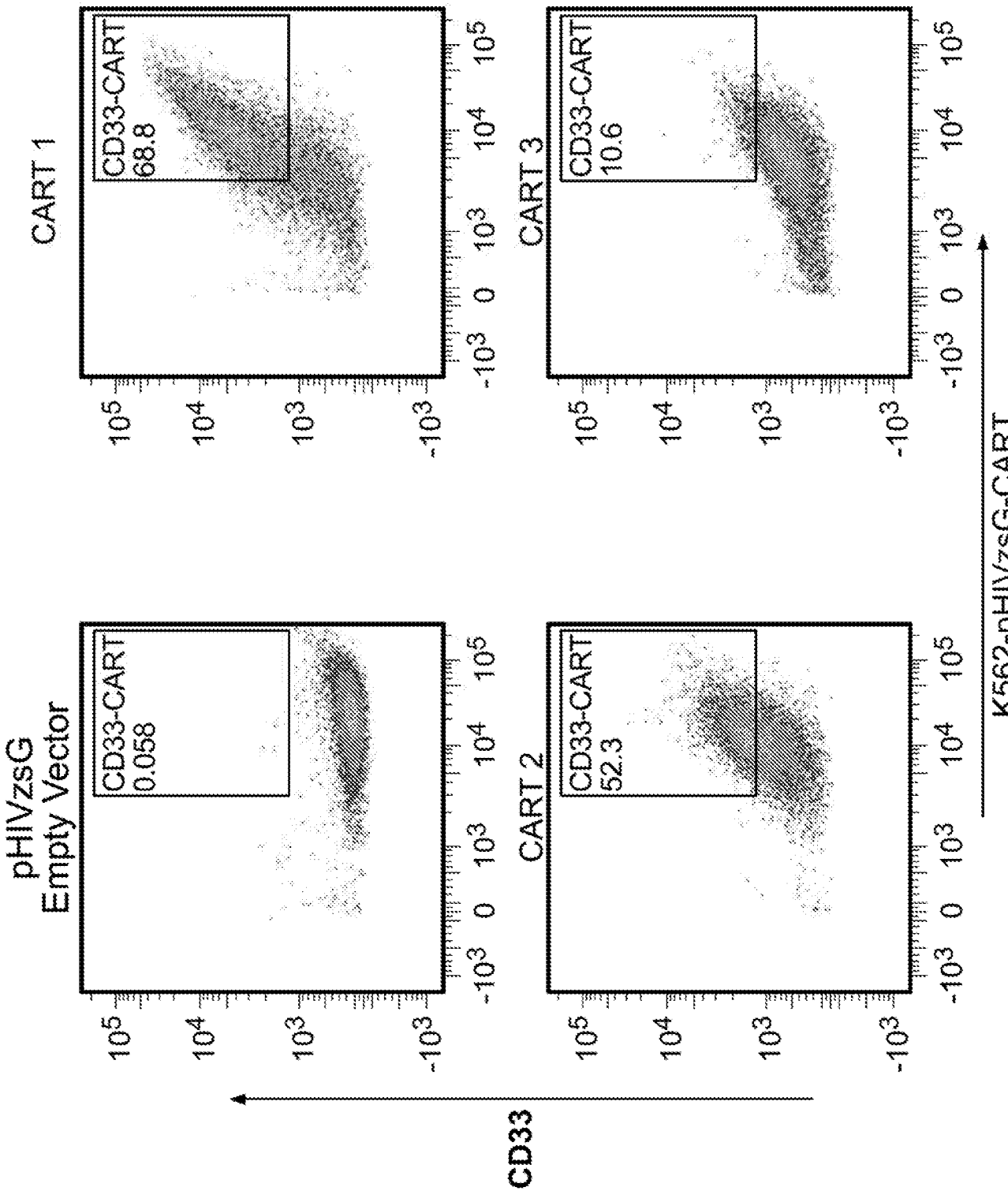

pHIVzsG
Empty Vector
CD33-CART
0.058
CART 1
CD33-CART
68.8
CART 2
CD33-CART
52.3
CART 3
CD33-CART
10.6
CD33
K562-pHIVzsG-CART

FIG. 10C

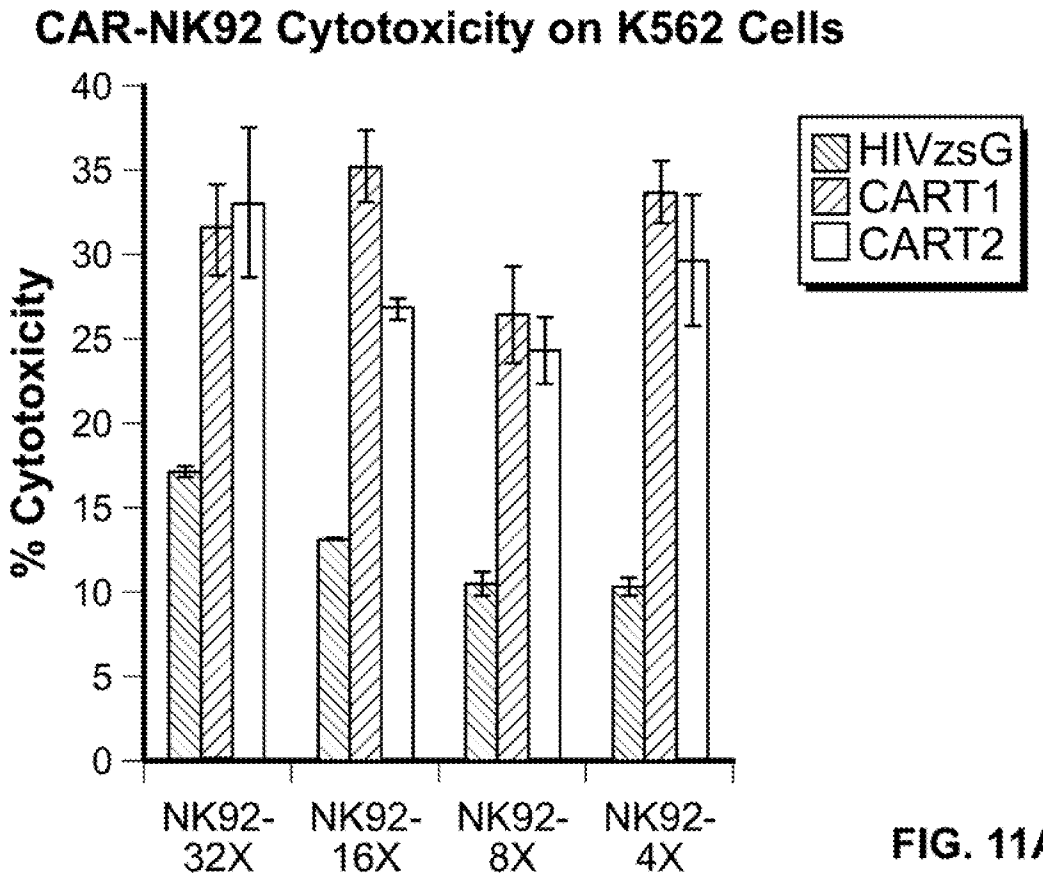


FIG. 11A

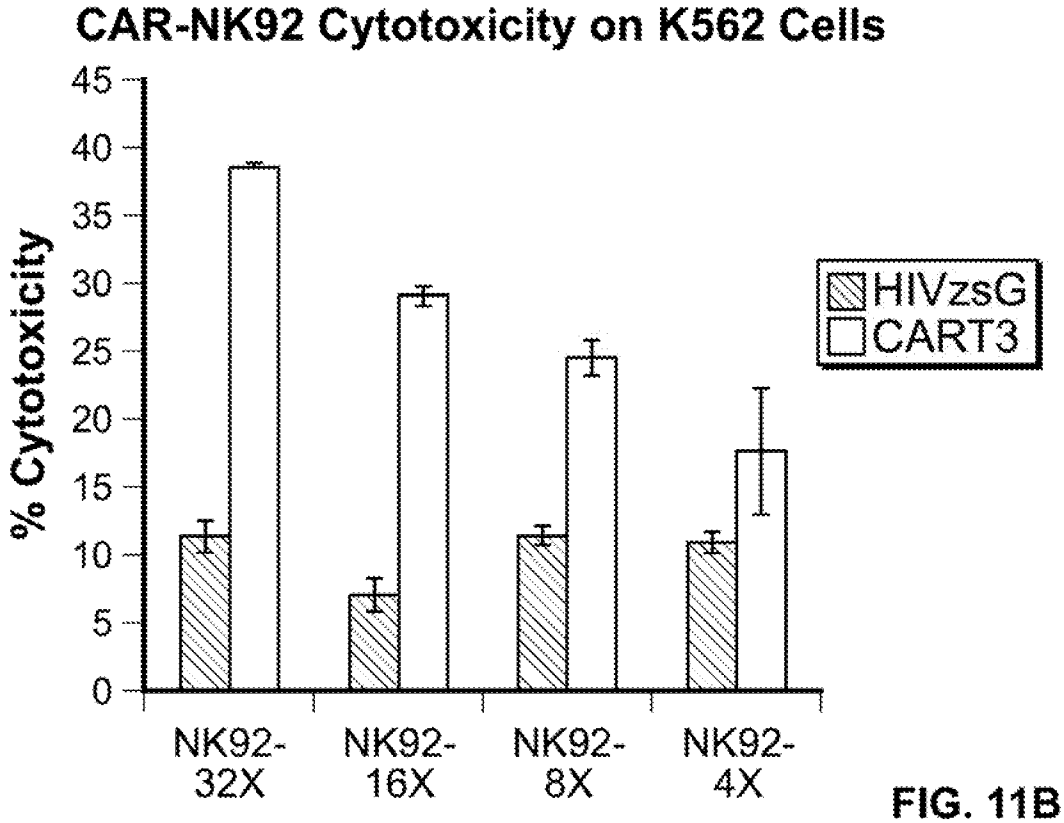


FIG. 11B

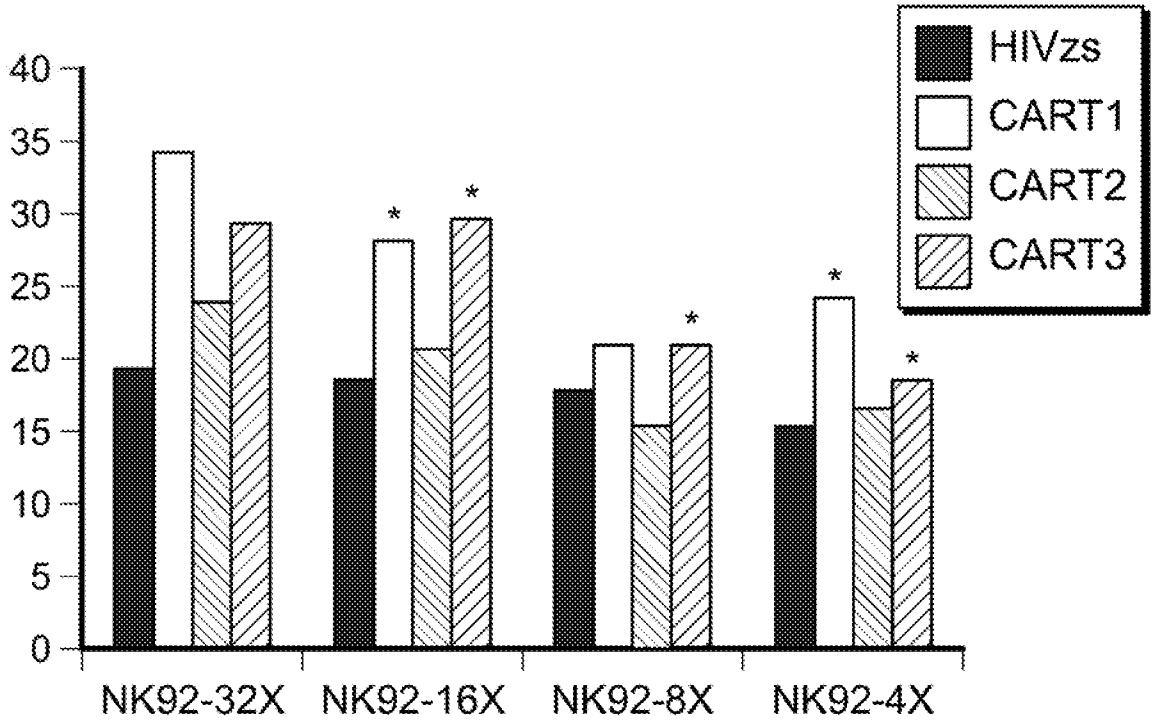


FIG. 12A

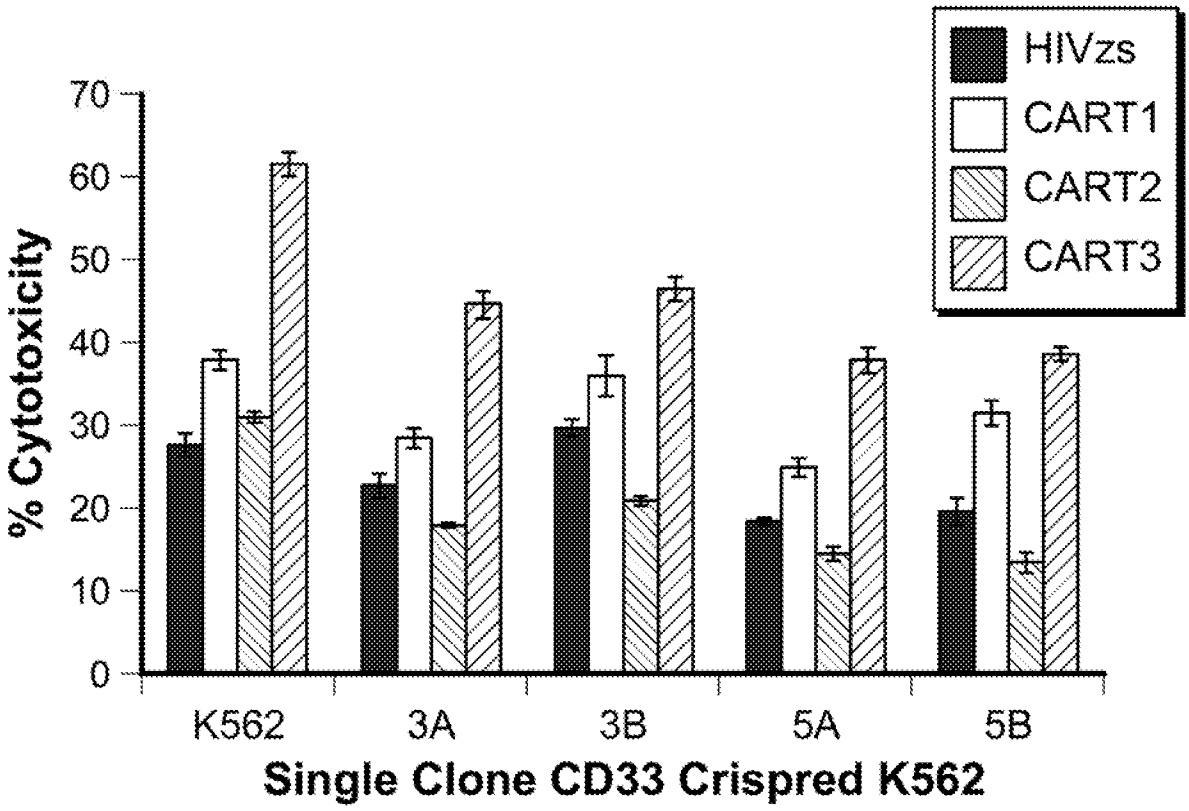


FIG. 12B

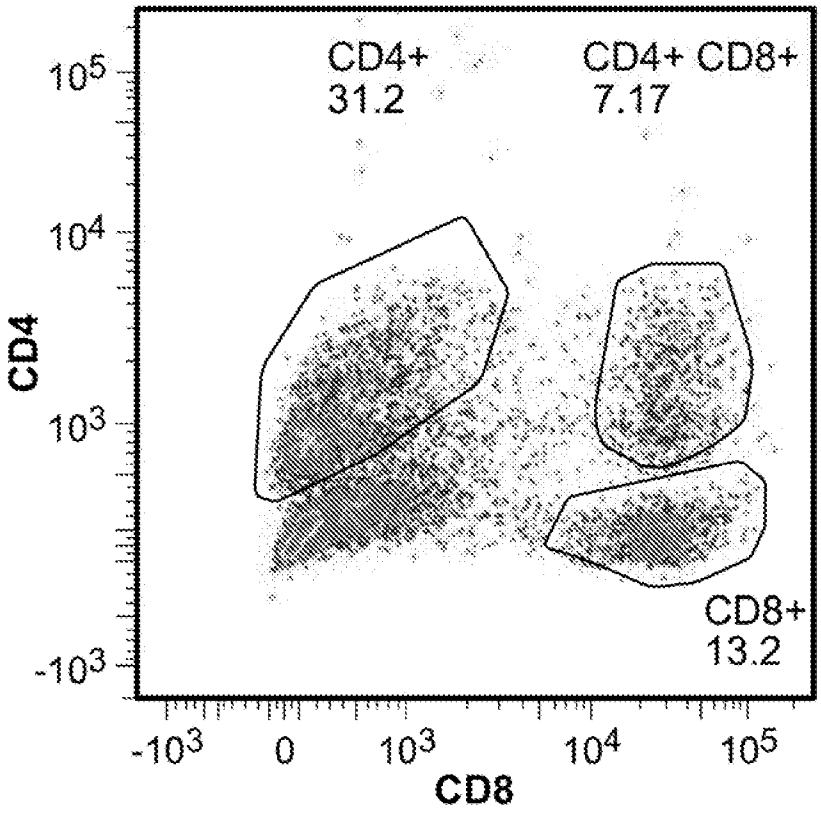


FIG. 13A

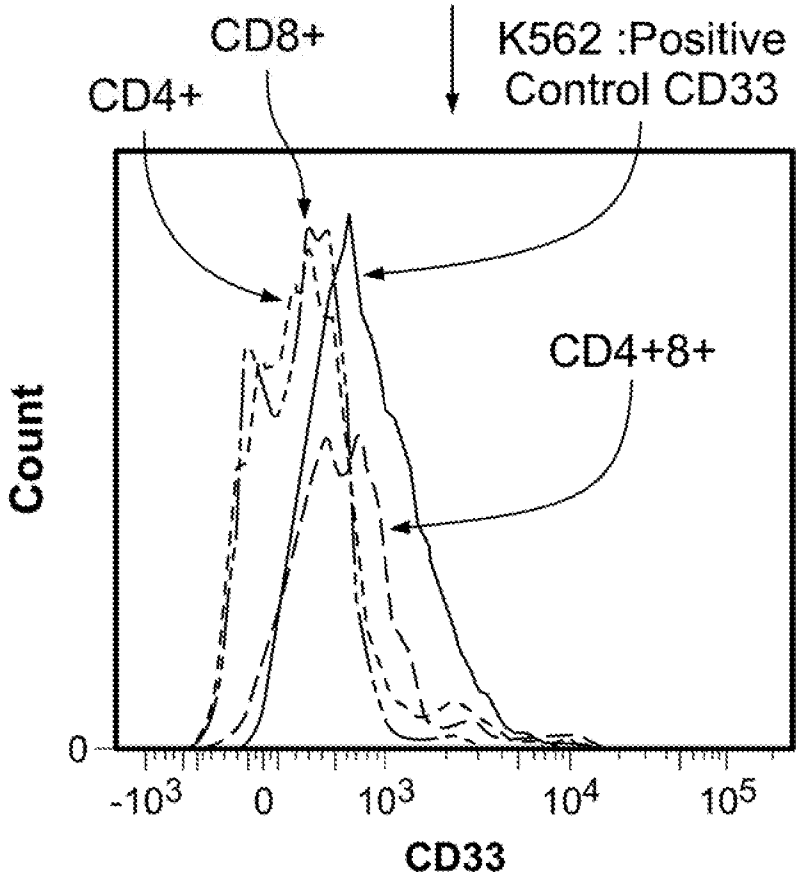


FIG. 13B

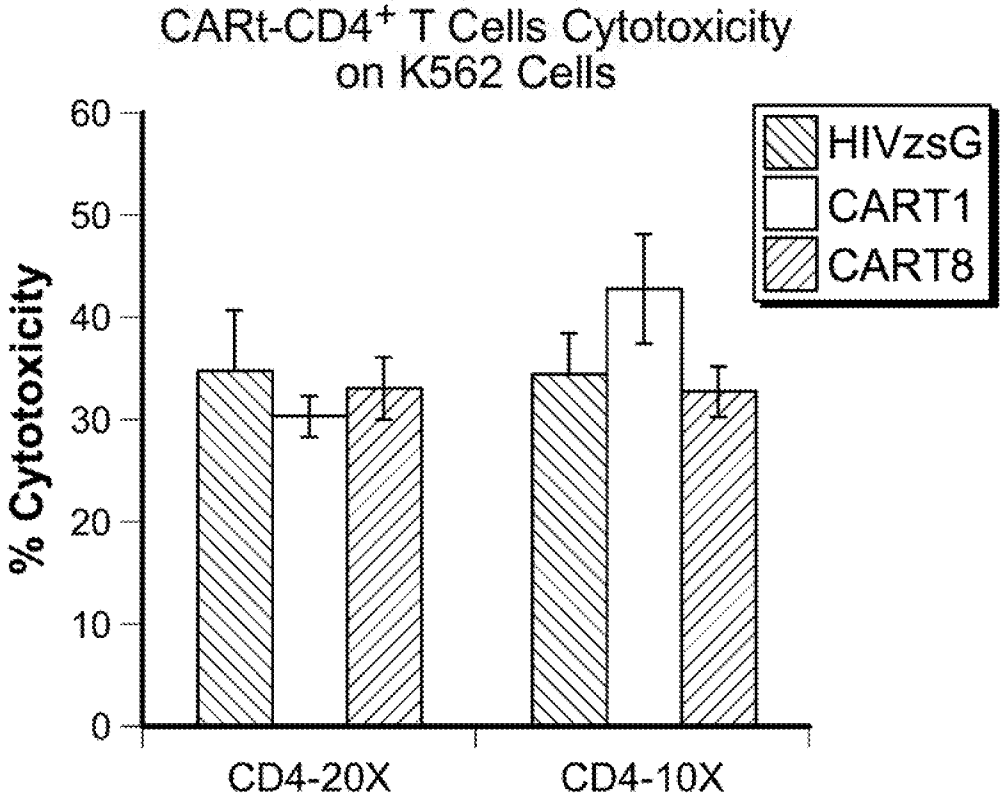


FIG. 14A

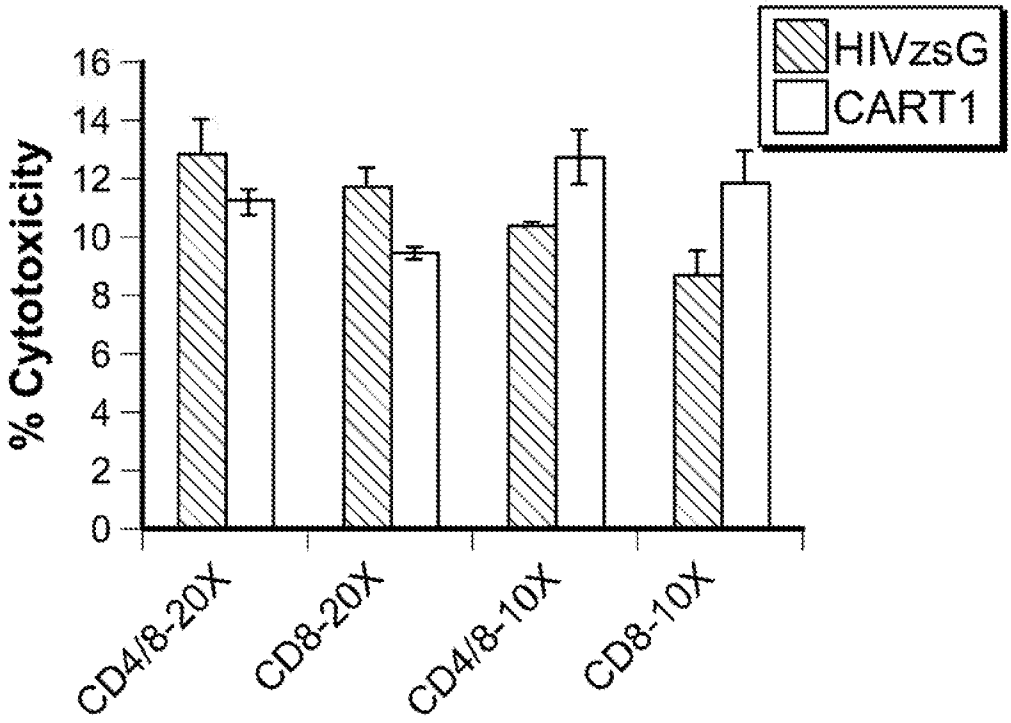


FIG. 14B

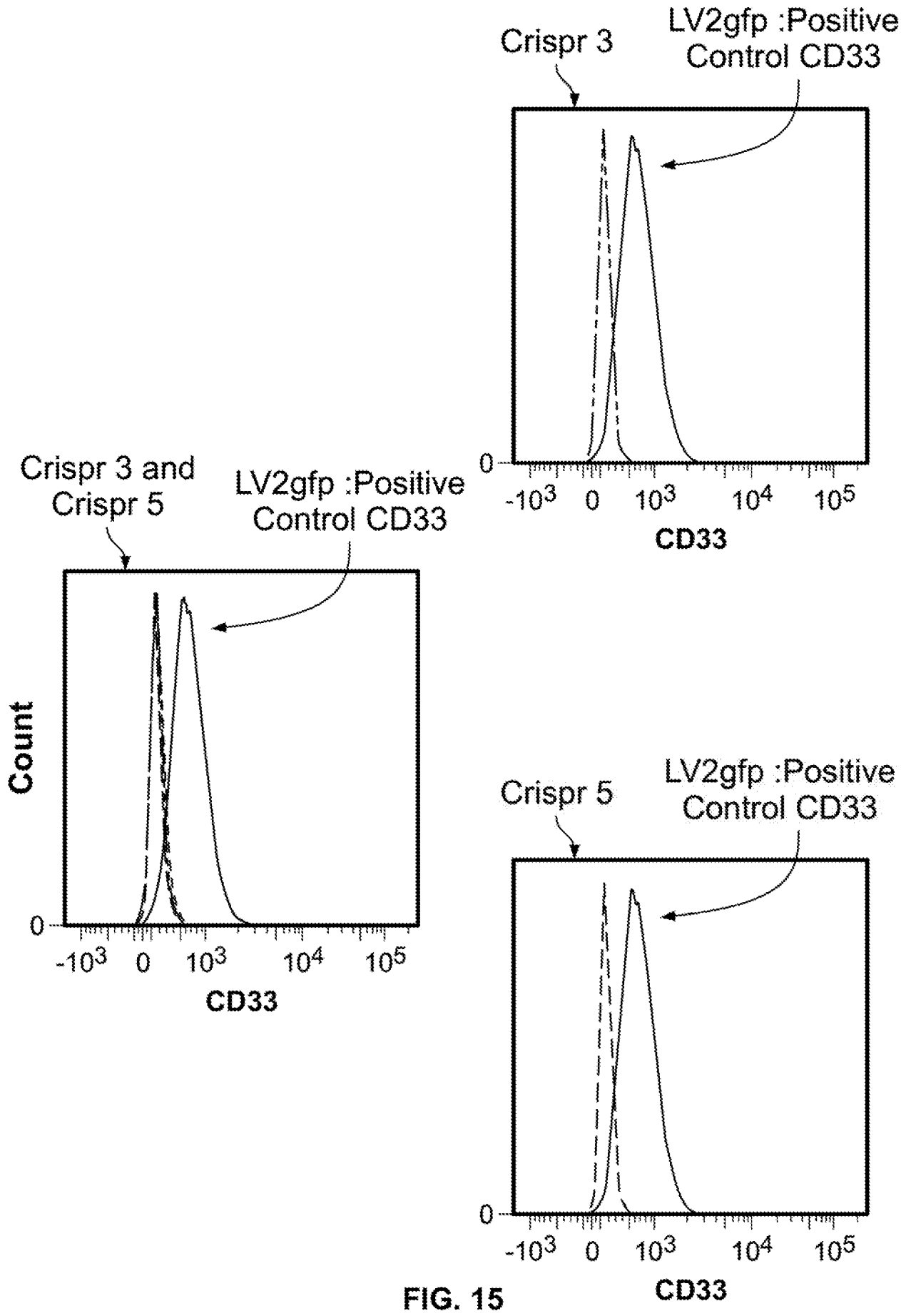

Crispr 3
LV2gfp :Positive Control CD33
0
-10^3 0 10^3 10^4 10^5
CD33
Crispr 3 and Crispr 5
LV2gfp :Positive Control CD33
Count
0
-10^3 0 10^3 10^4 10^5
CD33
Crispr 5
LV2gfp :Positive Control CD33
0
-10^3 0 10^3 10^4 10^5
CD33

FIG. 15

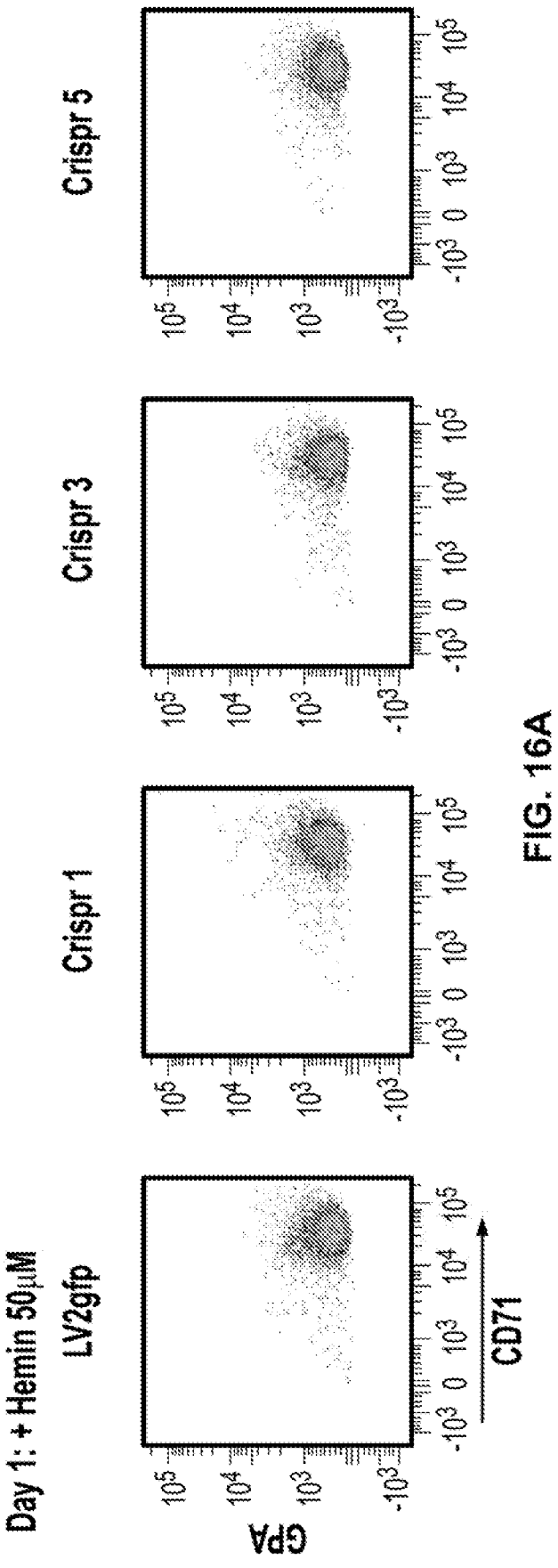


FIG. 16A

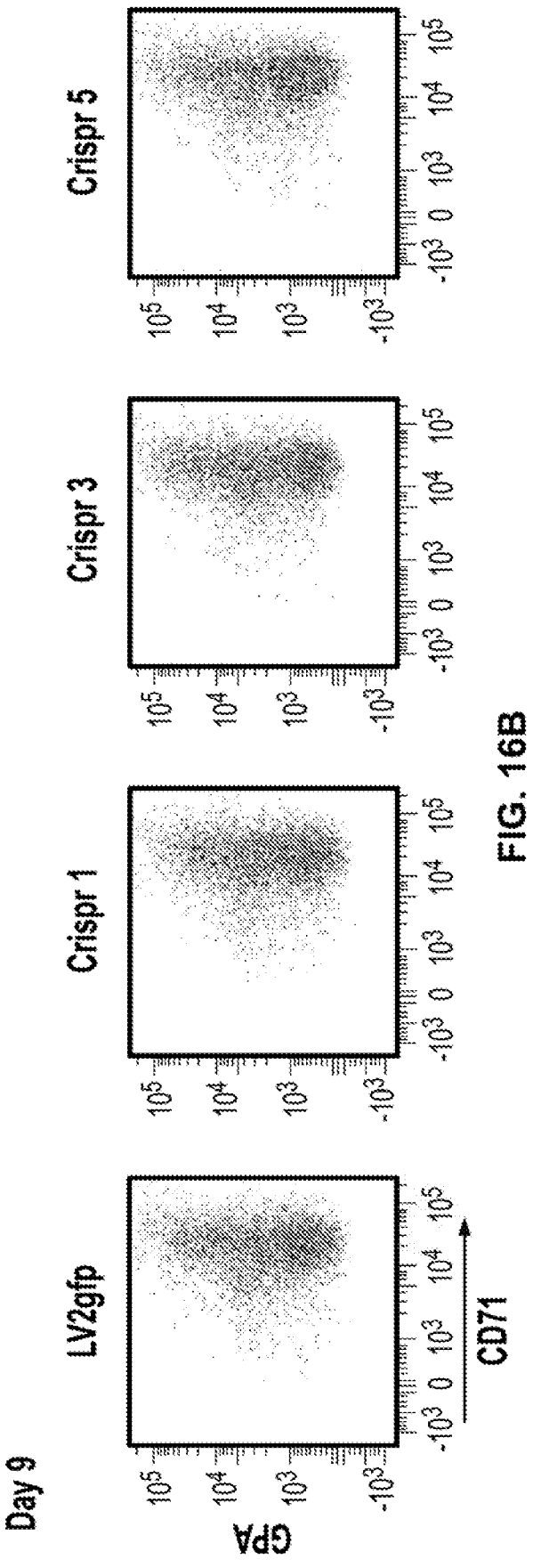


FIG. 16B

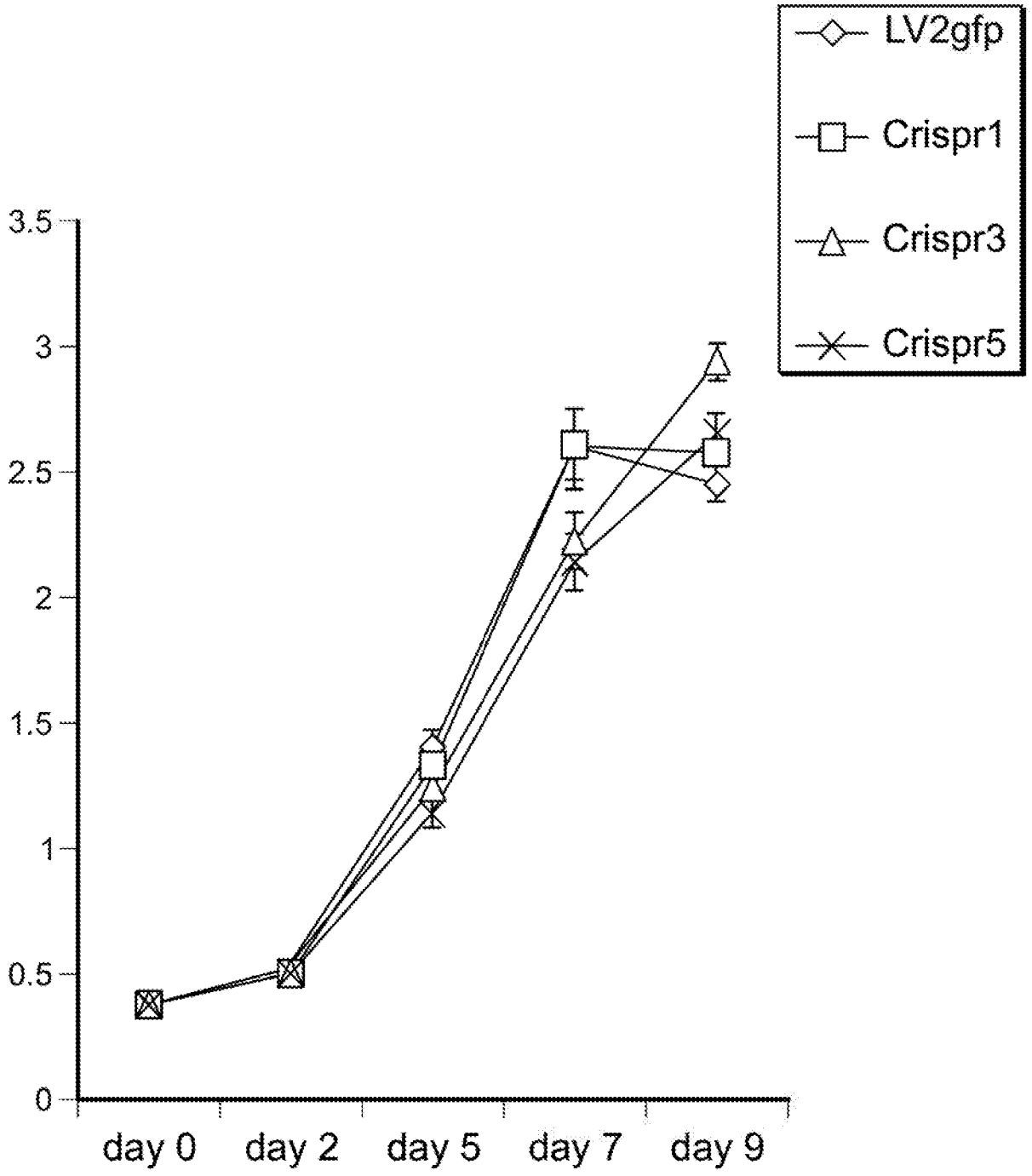

LV2gfp
Crispr1
Crispr3
Crispr5
3.5
3
2.5
2
1.5
1
0.5
0
day 0
day 2
day 5
day 7
day 9

FIG. 16C

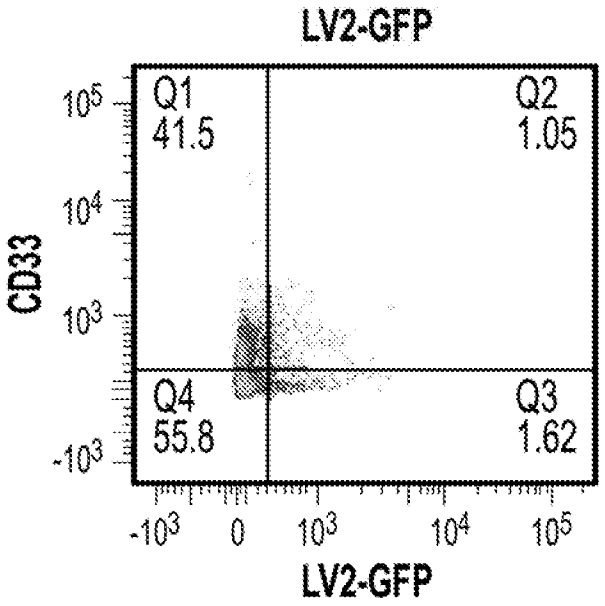


FIG. 17A

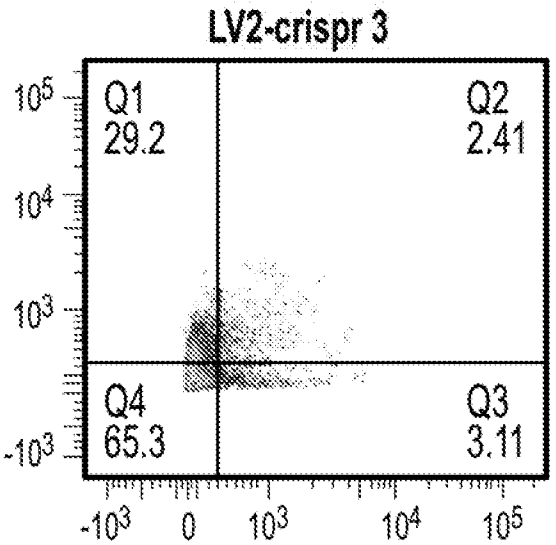


FIG. 17B

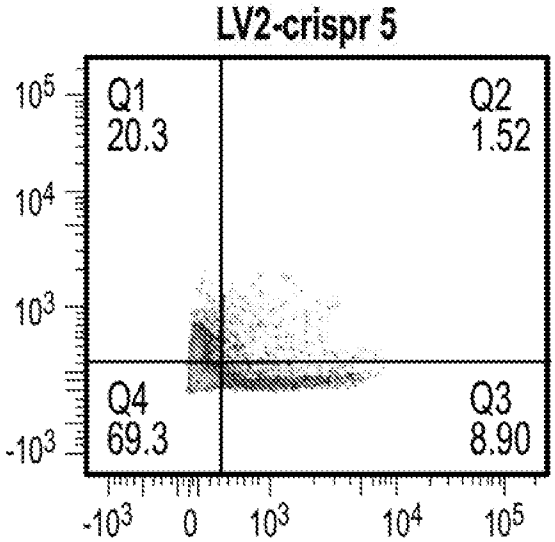


FIG. 17C

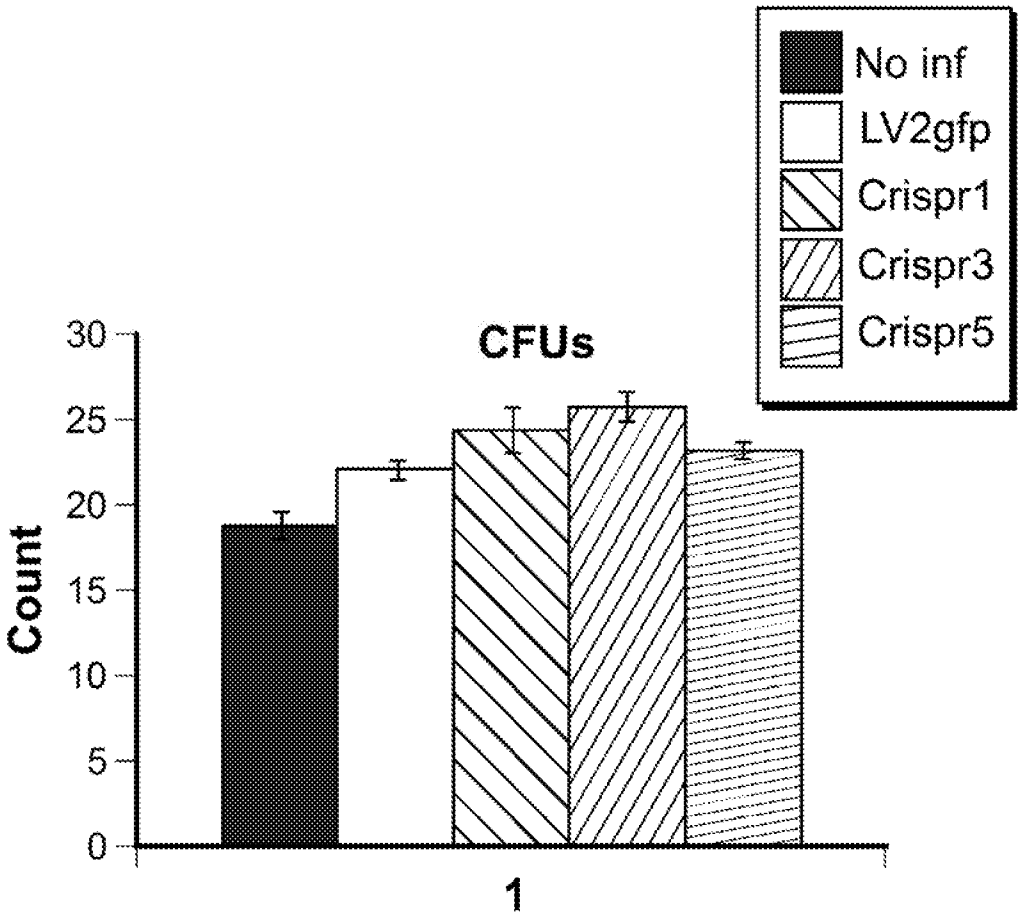

No inf
LV2gfp
Crispr1
Crispr3
Crispr5
CFUs
Count
30
25
20
15
10
5
0
1

FIG. 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/057339

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 12 and 13 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/057339

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

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

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

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

3. ☒ Claims Nos.: 4-26, 33-42, 49-51, 55-62
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/057339

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 14/705; C07K 14/725; C07K 16/28; C07K 19/00; C12N 5/0783 (2016.01)
CPC - A61K 35/17; C07K 14/70521; C07K 14/70596; C07K 2319/03 (2016.11)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC - A61K 31/436; A61K 35/17; C07K 14/705; C07K 14/71; C07K 14/725; C07K 16/28; C07K 19/00; C12N 5/0783; C12N 9/90
CPC - A61K 31/436; A61K 35/17; C07K 14/7051; C07K 14/70521; C07K 14/70539; C07K 14/70578; C07K 14/71; C07K 16/2863; C07K 14/70596; C07K 2317/622; C07K 2319/00; C07K 2319/03; C07K 2319/70; C12N 9/90; C12Y 502/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/23; 435/6.11; 435/7.92; 435/7.4; 435/7.23; 435/7.1; 506/9; 506/12 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google, PubMed
Search terms used: CD33 p67 SIGLEC-3 SIGLEC3 knockout deletion null Δ chimeric antigen receptor artificial t cell receptor chimeric t cell receptor chimeric immunoreceptor engineered t cell receptor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/090229 A1 (NOVARTIS AG et al) 25 June 2015 (25.06.2015) entire document	27-32
X -- Y	BRINKMAN-VAN DER LINDEN et al. "CD33/Siglec-3 Binding Specificity, Expression Pattern, and Consequences of Gene Deletion in Mice," Molecular and Cellular Biology, 01 June 2003 (01.06.2003), Vol. 23, No. 12, Pgs. 4199-4206. entire document	43-45, 47 --- 46, 48
X	ABRAHIMI et al. "Efficient Gene Disruption in Cultured Primary Human Endothelial Cells by CRISPR/Cas9," Circulation Research, 04 May 2015 (04.05.2015), Vol. 117, Iss. 2, Pgs. 121-128. entire document	52-54
Y	DOENCH et al. "Rational design of highly active sgRNAs for CRISPR-Cas9–mediated gene inactivation," Nature Biotechnology, 03 September 2014 (03.09.2014), Vol. 32, Pgs. 1262-1267 (Pgs. 1-17 for citations). entire document	46, 48
P, X	KIM et al. "Abstract 273: Genome Editing Using CRISPR-Cas9 to Increase the Therapeutic Index of Antigen-Specific Immunotherapy in Acute Myeloid Leukemia," American Society of Gene & Cell Therapy 19th Annual Meeting, Molecular Therapy, 04 May 2016 (04.05.2016), Vol. 24, Suppl. 1, Pg. S108. entire document	1-3, 43-48, 52-54
P, X	SOTILLO et al. "Convergence of Acquired Mutations and Alternative Splicing of CD19 Enables Resistance to CART-19 Immunotherapy," Cancer Discovery, 29 October 2015 (29.10.2015), Vol. 5, Iss. 12, Pgs. 1282-1295. entire document	1-3, 43-48, 52-54

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
28 December 2016	09 JAN 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/057339

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	KIM et al. "Engineering Resistance to Antigen-Specific Immunotherapy in Normal Hematopoietic Stem Cells by Gene Editing to Enable Targeting of Acute Myeloid Leukemia," American Society of Heamatology 58th Annual Meeting & Exposition, 05 December 2016 (05.12.2016), Pgs. 1-2. Retrieved from the Internet: <https://ash.confex.com/ash/2016/webprogram/Paper89763.html> on 28 December 2016 (28.12.2016). entire document	1-3, 43-48, 52-54