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(54) Title: IMMUNE CELLS COMPRISING A MODIFIED SUV39H1 GENE

(57) Abstract: The invention provides immune cells defective for SUV39H1 with enhanced properties



IMMUNE CELLS COMPRISING A MODIFIED SUV39H1 GENE

FIELD OF THE INVENTION

[0001] The present invention relates to the field of adoptive cell therapy. The present invention provides immune cells defective for SUV39H1 with enhanced properties.

5 BACKGROUND OF THE INVENTION

[0002] A variety of cancer therapies, including chemotherapy, antibody therapy, and adoptive cell therapy, are widely available. Yet they encounter hurdles such as cancers that are refractory or resistant to treatment, or cancers that relapse after an initial successful treatment.

[0003] Cell immunotherapy has been widely researched and several CAR T-cell therapies are commercially marketed for the treatment of B-cell or lymphoid malignancies. However, despite the impressive rates of initial complete responses observed with current CAR T-cell therapies for B-cell malignancies, longer term follow-up has demonstrated that a significant proportion of patients relapse after treatment. In addition, clinically effective treatment of solid tumors and myeloid malignancies has proven more challenging than treatment of lymphoid malignancies.

15 [0004] There remains a need for better or more effective treatments for cancer.

SUMMARY OF THE INVENTION

[0005] The present invention provides immune cells defective for SUV39H1 with enhanced properties.

[0006] In some embodiments, the antigen is a cancer antigen, notably a tumor specific antigen or a tumor associated antigen. Cancer antigens include orphan tyrosine kinase receptor ROR1, tEGFR, Her2, p95HER2, L1-CAM, CD19, CD20, CD22, mesothelin, CEA, Claudin 18.2, hepatitis B surface antigen, anti-folate receptor, a claudin (such as claudin 3, claudin 4, claudin 6 or claudin 18.2), CD23, CD24, CD30, CD33, CD38, CD44, EGFR, EGP-2, EGP-4, CD70, EPHA2, ErbB2, 3, or 4, FcRH5, FBP, fetal acetylcholine e receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-13R-alpha2, kdr, kappa light chain, BCMA, Lewis Y, MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, TAG72, VEGF- The present invention relates to the field of adoptive cell therapy. R2,

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carcinoembryonic antigen (CEA), prostate specific antigen (PSMA), estrogen receptor, progesterone receptor, ephrinB2, CD 123, CS-1, c-Met, GD-2, MAGE A3, CE7, or Wilms Tumor 1 (WT-1); or optionally any of the tumor neoantigenic peptides disclosed in Int'l Pat. Pub. No. WO 2021/043804. In some embodiments, the antigen is a neoantigen or a non-canonical antigen.

5 [0007] In some embodiments, the cancer is a myeloid cancer. In some embodiments, the cancer is a solid tumor. For example, the solid tumor is a cancer affecting an organ, optionally a cancer affecting colon, rectum, skin, endometrium, lung (including non-small cell lung carcinoma), uterus, bones (such as Osteosarcoma, Chondrosarcomas, Ewing's sarcoma, Fibrosarcomas, Giant cell tumors, Adamantinomas, and Chordomas), liver, kidney, esophagus, stomach, bladder, pancreas, cervix, brain (such as Meningiomas, Glioblastomas, Lower-Grade
10 Astrocytomas, Oligodendrocytomas, Pituitary Tumors, Schwannomas, and Metastatic brain cancers), ovary, breast (such as triple negative breast cancer), head and neck region, testis, prostate or the thyroid gland.

[0008] In some embodiments, the antigen-specific receptor is a TCR (which can be modified, recombinant or not), a chimeric antigen receptor (CAR) or a co-stimulatory receptor or ligand.
15 In some embodiments, the cell comprises at least one antigen-specific receptor having an intracellular signaling domain wherein one or two immunoreceptor tyrosine-based activation motifs (ITAMs) are inactivated, optionally wherein the antigen-specific receptor comprises a single active ITAM domain. For example, the antigen-specific receptor is a chimeric antigen
20 receptor (CAR) comprising: a) an extracellular antigen-binding domain that specifically binds an antigen, b) a transmembrane domain, c) optionally one or more costimulatory domains, and d) an intracellular signaling domain wherein one or two immunoreceptor tyrosine-based activation motifs (ITAMs) are inactivated; optionally wherein the antigen-specific receptor comprises a single active ITAM domain; and optionally wherein the intracellular domain comprises a
25 modified CD3zeta intracellular signaling domain in which ITAM2 and ITAM3 have been inactivated.

[0009] In some embodiments, the antigen-specific receptor comprises an extracellular antigen-binding domain which is an scFv; or a single domain antibody; or an antibody heavy chain region (VH) and/or an antibody variable region (VL); or optionally a bispecific or trispecific
30 antigen-binding domain. Optionally, the antigen-specific receptor comprises a transmembrane

domain from CD28, CD8 or CD3-zeta, or a fragment thereof. Optionally, the antigen-specific receptor comprises one or more costimulatory domains selected from the group consisting of: 4-1BB (CD137), CD28, CD27, ICOS, OX40 (CD134) and DAP10; DAP12, 2B4, CD40, FCER1G and/or GITR (AITR), or an active fragment thereof.

5 [0010] In some embodiments, the antigen-specific receptor comprises: a) an extracellular antigen-binding domain that specifically binds an antigen, optionally comprising an antibody heavy chain variable region and/or an antibody light chain variable region, and is optionally bispecific or trispecific; b) a transmembrane domain, optionally comprising a fragment of transmembrane domain of alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon,
10 CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD34, CD137, or CD154, NKG2D, OX40, ICOS, 2B4, DAP10, DAP12, CD40; c) optionally one or more co-stimulatory domains from 4-1BB, CD28, ICOS, OX40, DAP10 or DAP12, 2B4, CD40, FCER1G, or an active fragment thereof; and d) an intracellular signaling domain comprising an intracellular signaling domain from CD3zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon,
15 CDS, CD22, CD79a, CD79b, or CD66d, 2B4, or an active fragment thereof.

[0011] In some embodiments, the antigen-specific receptor is a chimeric antigen receptor (CAR) comprising: a) an extracellular antigen-binding domain, optionally an scFv, b) a transmembrane domain, optionally from CD28, CD8 or CD3-zeta, c) one or more co-stimulatory domains, optionally from 4-1 BB, CD28, ICOS, OX40 or DAP10, and d) an intracellular signaling
20 domain from CD3zeta, optionally in which ITAM2 and ITAM3 have been inactivated.

[0012] In some embodiments, the antigen-specific receptor is a modified TCR that comprises a heterologous extracellular antigen-binding domain that specifically binds an antigen, optionally comprising an antibody heavy chain variable region and/or an antibody light chain variable region, and is optionally bispecific or trispecific. Optionally, the antigen-specific receptor is a
25 modified TCR that comprises a fragment of an alpha, beta, gamma or epsilon chain and a heterologous extracellular antigen-binding domain that specifically binds an antigen, optionally comprising one or more single domain antibody, or an antibody heavy chain variable region and/or an antibody light chain variable region, optionally an scFv, and is optionally bispecific or trispecific. In some embodiments, modified TCR can be named recombinant HLA-independent
30 (or non-HLA restricted) T cell receptors (referred to as "HI-TCRs") that bind to an antigen of

interest in an HLA-independent manner. HLA independent (modified) TCRs are notably described in International Application No. WO 2019/157454. Such HI-TCRs comprise an antigen binding chain that comprises: (a) an antigen-binding domain that binds to an antigen in an HLA-independent manner, for example, an antigen-binding fragment of an immunoglobulin variable region; and (b) a constant domain that is capable of associating with (and consequently activating) a CD3 ζ polypeptide. Because typically TCRs bind antigen in a HLA-dependent manner, the antigen-binding domain that binds in an HLA-independent manner must be heterologous.

[0013] The antigen-binding domain or fragment thereof comprises: a single domain antibody (VHH), or a heavy chain variable region (VH) of an antibody and/or a light chain variable region (VL) of an antibody. The constant domain of the TCR is, for example, a native or modified TRAC polypeptide, or a native or modified TRBC polypeptide. The constant domain of the TCR is, for example, a native TCR constant domain (alpha or beta) or fragment thereof. Unlike chimeric antigen receptors, which typically themselves comprise an intracellular signaling domain, the HI-TCR does not directly produce an activating signal; instead, the antigen-binding chain associates with and consequently activates a CD3 ζ polypeptide. The immune cells comprising the recombinant TCR is highly sensitive and typically provide high activity when the targeted antigen is expressed at a low density (typically of less than about 10,000 molecules per cell) on the surface of a cell.

[0014] In some embodiments, the antigen-specific receptor is thus a modified TCR that comprises: a) a first antigen-binding chain comprising an antigen-binding fragment of a heavy chain variable region (VH) of an antibody; and b) a second antigen-binding chain comprising an antigen-binding fragment of a light chain variable region (VL) of an antibody; wherein the first and second antigen-binding chains each comprise a TRAC polypeptide or a TRBC polypeptide, optionally wherein at least one of the TRAC polypeptide and the TRBC polypeptide is endogenous, and optionally wherein one or both of the endogenous TRAC and TRBC polypeptides is inactivated.

[0015] In some embodiments, a heterologous nucleic acid sequence encoding the antigen-specific receptor or a portion thereof is inserted into the cell genome to express the antigen-

specific receptor. In other embodiments, a heterologous nucleic acid sequence outside the cell genome expresses the antigen-specific receptor.

[0016] In some embodiments, insertion of the heterologous nucleic acid sequence encoding the antigen-specific receptor or a portion thereof inactivates expression of a native TCR alpha chain and/or a native TCR beta chain. In any of these embodiments, expression of the antigen-specific receptor may be under control of an endogenous promoter of a TCR, optionally an endogenous TRAC promoter.

[0017] In any of these embodiments, the immune cell is a T cell, a CD4⁺ T cell, a CD8⁺ T cell, a CD4⁺ and CD8⁺ T cell, a NK cell, a T regulatory cell, a TN cell, a memory stem T cell (TSCM), a TCM cell, a TEM cell, a monocyte, a dendritic cell, or a macrophage, or a progenitor thereof, optionally a T cell progenitor, a lymphoid progenitor, an NK cell progenitor, a myeloid progenitor, a pluripotent stem cell, an induced pluripotent stem cell (iPSC), a hematopoietic stem cell (HSC), an adipose derived stem cell (ADSC), or a pluripotent stem cell of myeloid or lymphoid lineage. Optionally, the immune cell is a T cell or NK cell, or progenitor thereof.

[0018] In any of these embodiments, the modified immune cell may further comprise a second engineered antigen-specific receptor, optionally a modified TCR or CAR, that specifically binds to a second antigen. For example, the modified immune cell comprises a first CAR that binds the antigen and a second CAR that binds a second antigen. As another example, the modified immune cell comprises a CAR that binds the antigen and modified TCR that binds a second antigen. As another example, the modified immune cell comprises a first modified TCR that binds the antigen and a second modified TCR that binds a second antigen. In some embodiments, the modified immune cell comprises three or more engineered antigen-specific receptors.

[0019] In any of these embodiments, the modified immune cell may further comprise a heterologous co-stimulatory receptor. In some embodiments, the co-stimulatory receptor comprises (a) an extracellular domain of a co-stimulatory ligand, optionally from CD80, (b) a transmembrane domain, optionally from CD80, and (c) an intracellular domain of a co-stimulatory molecule, optionally CD28, 4-1BB, OX40, ICOS, DAP10, CD27, CD40, NKGD2, or CD2, preferably 4-1BB.

[0020] In some embodiments, the extracellular antigen-binding domain binds, or comprises a VH and/or VL from an antibody that binds, an antigen with a KD affinity of about 1×10^{-7} M or less, about 5×10^{-8} M or less, about 1×10^{-8} M or less, about 5×10^{-9} M or less, about 1×10^{-9} M or less, about 5×10^{-10} M or less, about 1×10^{-10} M or less, about 5×10^{-11} M or less, about 1×10^{-11} M or less, about 5×10^{-12} M or less, or about 1×10^{-12} M or less (lower numbers indicating greater binding affinity).

[0021] In some embodiments, the antigen has a low density on the cell surface, of less than about 10,000, or less than about 5,000, or less than about 2,000 molecules per cell.

[0022] In some embodiments, SUV39H1 expression in the cell is reduced or inhibited by at least about 50%, 60%, 70%, 75%, 80%, 85%, 90% or 95%. In some embodiments, endogenous TCR expression of the cell is reduced by at least about 75%, 80%, 85%, 90% or 95%.

[0023] The modified immune cell may be allogeneic or autologous. For example, an HLA-A locus is inactivated in the cell. In some embodiments, HLA class I expression is reduced by at least about 75%, 80%, 85%, 90% or 95%.

[0024] In another aspect, the disclosure provides a method of producing the foregoing modified immune cell(s) comprising

[0025] Disclosed herein are also methods for improving the memory function and/or survival and/or fitness of immune cell to be used in cell therapy.

[0026] The present invention therefore encompasses a method for producing a genetically modified immune cell comprising a modified Suv39H1 region gene, said method comprising: introducing into an immune cell:

- a first nucleic acid sequence comprising a first exogenous polynucleotide encoding an engineered nuclease protein, or (ii) an engineered nuclease protein, wherein said engineered nuclease produces a cleavage site at a recognition sequence within said Suv39h1 region gene; and
- a second nucleic acid sequence comprising a second exogenous polynucleotide encoding a therapeutic protein.

[0027] In some embodiments, the nuclease is a CRISPR Cas9 nuclease. In some embodiment, the method further comprises introducing a guide RNA specific for Suv39h1, notably a guide RNA as herein disclosed.

[0028] The invention further comprises a method for producing a genetically modified immune cell comprising a modified human Suv39H1 region gene, said method comprising:

introducing into a cell: a first nucleic acid sequence encoding an engineered nuclease; or an engineered nuclease protein; wherein said engineered nuclease produces a cleavage site at a recognition sequence within said human Suv39h1 region gene; and

introducing into said cell a second nucleic acid sequence comprising an exogenous polynucleotide; wherein the sequence of said exogenous polynucleotide is inserted into said Suv39h1 region gene at said cleavage site; and further wherein said genetically-modified immune cell has reduced or abolished expression of a functional Suv39H1 protein when compared to an unmodified control immune cell. In some embodiments, the nuclease is a CRISPR Cas9 nuclease.

In some embodiment, the method further comprises introducing a guide RNA specific for Suv39h1, notably a guide RNA as herein disclosed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] Fig. 1. a. The table shows the target exon of different SUV39H1-targeting gRNAs. b. Schematic of Crispr/Cas9-targeting of SUV39H1 locus. Top: SUV39H1 locus around exon 2. Bottom: AAV6 vector containing the chimeric antigen receptor expression cassette, which contains a splice acceptor, a P2A peptide, the CAR transgene (e.g. a 1928z CAR) and a poly-A tail. The CAR cassette is flanked by homology arms. c. Schematic of Crispr/Cas9-targeting of SUV39H1 locus. Top: SUV39H1 locus around exon 2. Bottom: AAV6 vector containing the chimeric antigen receptor expression cassette, which is antiparallel and contains an EF1a promoter, the CAR transgene (e.g., a 1928z CAR) and a poly-A tail. The CAR cassette is flanked by homology arms.

[0030] Fig. 2. a. Schematic of the experimental procedure of SUV39H1-KO – CAR-KI using the antiparallel CAR expression cassette. T cell activation was performed on day 0, Cas9 ribonucleoparticle (RNP) nucleofection was performed on day 3, followed a few hours later

infection with AAV particles. On day 7, the cells were harvested, and then analysed for expression of CAR, SUV39H1, and the memory marker CD27, as well as the tri-methylation of H3K9. b. Expression of SUV39H1 by western blotting in T cells treated with different multiplicities of AAV infection, in the absence (Mock) or presence (gRNA SUV) of a SUV39H1-targeting gRNA. Results are shown for a representative donor. c. Levels of tri-methylation of H3K9 by flow cytometry (geometric mean fluorescence intensity) in T cells treated with a 500000-multiplicity of AAV infection, in the absence (Mock) or presence (gRNA SUV) of a SUV39H1-targeting gRNA. Each data point represents a different donor (n=2). d. Expression by flow cytometry of the memory marker CD27 in T cells treated with a 500000-multiplicity of AAV infection, in the absence (Mock) or presence (gRNA SUV) of a SUV39H1-targeting gRNA. Results are shown for a representative donor.

[0031] Fig. 3. a. Percentage of CAR expression by flow cytometry in T cells treated with different multiplicities of AAV infection, in the absence (Mock) or presence (gRNA SUV) of a SUV39H1-targeting gRNA. Results are shown for a representative donor. b. Expression levels by flow cytometry (geometric mean fluorescence intensity) in T cells treated with a 500000-multiplicity of AAV infection, in the absence (Mock) or presence (gRNA SUV) of a SUV39H1-targeting gRNA. Each data point represents a different donor (n=2).

DETAILED DESCRIPTION OF THE INVENTION

[0032] The present application relates to immunotherapy and specifically to targeted cell therapies based on genetically engineering immune cells to express a therapeutic transgene under desired conditions. Described herein is a method for generating immune cells for immunotherapy by targeting the integration of a therapeutic transgene into the genome of an immune cell such that the transgene is inserted at a site of the said immune cell genome that reduces or prevents Suv39H1 expression. It will be understood that reference to a transgene (in the singular) as described herein applies also to one or more transgenes (in the plural) unless context indicates otherwise.

[0033] The presently disclosed subject matter further provides immune cells comprising a recombinant therapeutic protein, typically a chimeric antigen receptor or an exogenous TCR, notably a modified TCR expressed at the cell surface.

[0034] SUV39H1 is a H3K9-histone methyltransferase that plays a role in silencing memory and stem cell programs during the terminal differentiation of effector CD8⁺ T cells. Silencing of SUV39H1, in turn, has been shown to enhance long-term memory potential and to increase survival capacity. Herein is notably described a method for a one-step generation of an engineered immune cell to be used in cell therapy and having improved memory function and increase survival capacity by targeting the integration of a transgene cassette (i.e. an exogenous nucleic acid), into the Suv39H1 gene such that the integration of the cassette disrupts or reduces its expression. The method is suitable with the commonly used genome editing platforms, such as zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), clustered regularly-interspersed short palindromic repeats (CRISPR) associated nuclease such as type II Cas (Cas 9) or type V (notably type Va or Vb such as *cpf1* or *c2c1*) and variants thereof, Meganuclease or a Mega-Tal, and results in homologous recombination at a target site in the genome of the cell. The present application also provides an all in one method for producing an modified immune cell for cell therapy expressing a therapeutic protein such as a CAR or a modified TCR having improved memory function, increase persistence, improved fitness and/or increase survival capacity after adoptive cell transfer, wherein the said therapeutic protein is inserted at a SUV39 gene loci, as compared to a control immune cell comprising expressing the said therapeutic protein but having a non-modified SUV39H1 gene.

[0035] Exogenous nucleic acids or transgene cassettes mentioned above can encode, for example, a chimeric antigen receptor, a modified or exogenous TCR receptor, or any other therapeutic polypeptide of interest. Thus, the present invention allows for both the knockout of SUV39H1 and the expression of an exogenous nucleic acid sequence (e.g., a chimeric antigen receptor or modified/exogenous TCR) by targeting a single recognition site within the Suv39H1 gene with a single engineered nuclease.

[0036] In some embodiments, expression of the endogenous TCR is further disrupted. Such cells can exhibit reduced or no induction of graft-versus-host-disease (GVHD) when administered to an allogeneic subject.

Definitions

[0037] As used herein, the singular forms “a,” “an,” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0038] Also as used herein, “and/or” refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (“or”).

[0039] The term “about,” as used herein when referring to a measurable value such as an amount of polypeptide, dose, time, temperature, enzymatic activity or other biological activity and the like, is meant to encompass variations of $\pm 10\%$, $\pm 5\%$, $\pm 1\%$, $\pm 0.5\%$, or even $\pm 0.1\%$ of the specified amount.

[0040] The term “antibody” herein is used in the broadest sense and includes polyclonal and monoclonal antibodies, including intact antibodies and functional (antigen-binding) antibody fragments, including fragment antigen binding (Fab) fragments, F(ab')₂ fragments, Fab' fragments, Fv fragments, recombinant IgG (rIgG) fragments, variable heavy chain (VH) regions capable of specifically binding the antigen, single chain antibody fragments, including single chain variable fragments (scFv), and single domain antibodies (e.g., sdAb, sdFv, nanobody) fragments. The term encompasses recombinant and/or otherwise modified forms of immunoglobulins, such as intrabodies, peptibodies, chimeric antibodies, fully human antibodies, humanized antibodies, and heteroconjugate antibodies, multispecific, e.g., bispecific, antibodies, diabodies, triabodies, and tetrabodies, tandem di-scFv, tandem tri-scFv. Unless otherwise stated, the term “antibody” should be understood to encompass functional antibody fragments thereof. The term also encompasses intact or full-length antibodies, including antibodies of any class or sub-class, including IgG and sub-classes thereof, IgG1, IgG2, IgG3, IgG4, IgM, IgE, IgA, and IgD. In some embodiments the antibody comprises a heavy chain variable region and a light chain variable region.

[0041] An “antibody fragment” refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')₂; diabodies; linear antibodies; variable heavy chain (VH) regions, VHH antibodies, single-chain antibody molecules such as scFvs and single-domain VH single antibodies; and multispecific antibodies formed from antibody fragments. In particular embodiments, the antibodies are single-chain antibody fragments comprising a variable heavy chain region and/or a variable light chain region, such as scFvs.

[0042] “Inactivation” or “disruption” of a gene refers to a change in the sequence of genomic DNA that causes the gene’s expression to be reduced or eliminated, or that cause a non-functional gene product to be expressed. Exemplary methods include gene silencing, knockdown, knockout, and/or gene disruption techniques, such as gene editing through, e.g., induction of breaks and/or homologous recombination. Exemplary of such gene disruptions are insertions, frameshift and missense mutations, deletions, knock-in, and knock-out of the gene or part of the gene, including deletions of the entire gene. Such disruptions can occur in the coding region, e.g., in one or more exons such as by insertion of a stop codon, as well as in non-coding region, e.g., in the promoter, enhancer or other region affecting activation of transcription to prevent transcription of the gene, or in intron regions (e.g., by introduction of a frameshift) resulting in the inability to produce a full-length product and/or functional product, or any product. In some cases, a truncated gene disruption involves gene targeting, including targeted gene inactivation by homologous recombination.

[0043] Inactivation or disruption of a gene (the SUV39H1 gene) can decrease the expression of the gene product (e.g., the SUV39H1 methyl transferase) by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more as compared to the protein expression level associated with the wildtype gene.

[0044] Inactivation or disruption of a gene can decrease the activity of the expressed gene product (e.g., the SUV39H1 methyl transferase) by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or as compared to the protein expression level associated with the wildtype gene.

[0045] As used herein, “inhibition” or “reduced expression” of a gene product (e.g., the SUV39H1 protein refers to a decrease of its activity (e.g., the reduction of the activity of the gene product such as the Suv39H1 methyl transferase) and/or of the gene expression of at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more as compared to the activity or expression levels of wildtype which is not inhibited or repressed. The term reduced can also refer to a reduction in the percentage of cells in a population of cells that express an endogenous polypeptide (e.g. the Suv39H1 methyl transferase) at the cell surface when compared to a population of control cells. Such a reduction may be up to 5%, 10%, 20%, 30%, 40%, 50%, 60%,

70%, 80%, 90%, 95%, or up to 100%. Accordingly, the term “reduced” encompasses both a partial knockdown and a complete knockdown of the endogenous T cell receptor.

[0046] As used herein, “express” or “expression” means that a gene sequence is transcribed, and optionally, translated. If the gene expresses a noncoding RNA, expression will typically result in an RNA after transcription and, optionally, splicing. If the gene is a coding sequence, expression will typically result in production of a polypeptide after transcription and translation.

[0047] As used herein, a “vector” is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell.

[0048] As used herein, “expression control sequence” refers to a nucleotide sequence that influences the transcription, RNA processing, RNA stability, or translation of the associated nucleotide sequence. Examples include, but are not limited to, promoters, enhancers, introns, translation leader sequences, polyadenylation signal sequences, transcription initiators and transcriptional and/or translational termination region (i.e., termination region).

[0049] As used herein, “fragment” means a portion of a referenced sequence (polynucleotide or polypeptide) that has a length at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the full-length sequence.

[0050] As used herein, “heterologous” refers to a polynucleotide or polypeptide that comprises sequences that are not found in the same relationship to each other in nature. For example, the heterologous sequence either originates from another species, or is from the same species or organism but is modified from either its original form or the form primarily expressed in the cell. Thus, a heterologous polynucleotide includes a nucleotide sequence derived from and inserted into the same natural, original cell type, but which is present in a non-natural state, e.g., a different copy number, and/or under the control of different regulatory sequences than that found in nature and/or located in a different position (adjacent to a different nucleotide sequence) than where it was originally located.

[0051] As used herein, “nucleic acid,” “nucleotide sequence,” and “oligonucleotide” or “polynucleotide” are used interchangeably and encompass both RNA and DNA, including cDNA, genomic DNA, mRNA, synthetic (e.g., chemically synthesized) DNA or RNA and chimeras of RNA and DNA. The term polynucleotide, nucleotide sequence, or nucleic acid refers to a chain of nucleotides without regard to length of the chain. The nucleic acid can be double-stranded or

single-stranded. Where single-stranded, the nucleic acid can be a sense strand or an antisense strand. The nucleic acid can be synthesized using oligonucleotide analogs or derivatives (e.g., inosine or phosphorothioate nucleotides). Such oligonucleotides can be used, for example, to prepare nucleic acids that have altered base-pairing abilities or increased resistance to nucleases.

5 The present disclosure further provides nucleic acid inhibitors that are complementary to a nucleic acid, nucleotide sequence, or polynucleotide described herein. Modified bases (modified nucleobases), such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others can also be used for antisense, dsRNA, and ribozyme pairing. For example, polynucleotides that contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high
10 affinity and to be potent antisense inhibitors of gene expression. Other modifications, such as modification to the phosphodiester backbone, or the 2'-hydroxy in the ribose sugar group of the RNA can also be made.

[0052] As used herein, the term “exogenous protein” or “exogenous nucleic acid” refers to a protein or nucleic acid that is not found in the cell or a protein or nucleic acid that is not normally
15 found at the targeted genomic location but otherwise present in the cell.

[0053] Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins, which provide bacteria with adaptive immunity to foreign nucleic acids, have been repurposed for use in targeted genome editing in human cells and other types of cells, as well as in animals and plants. The CRISPR/Cas9 technology originates from type II
20 CRISPR/Cas systems, which consist of one DNA endonuclease protein, Cas9, and two small RNAs, CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The small RNAs or a chimeric single guide RNA (sgRNA) bind Cas9, thus forming an RNA-guided DNA endonuclease (RGEN) complex and cleave a specific DNA target. Chromosomal double-strand blunt-end breaks (DSBs) are then repaired via homologous recombination (HR) or non-
25 homologous end-joining (NHEJ) and produce genetic modifications. Suv-specific sgRNA are described below.

[0054] Type V-A (e.g., Cpf1), type V-C, and type V-D CRISPR-Cas systems naturally include a Cas nuclease and a single guide RNA (i.e., crRNA). In particular, Cpf1 is another type of RGEN derived from the type V CRISPR system which differs from Cas9 in several ways. First,
30 the function of Cpf1 requires only a crRNA, rather than a crRNA/tracrRNA pair. Second, Cas9

cleavage results in blunt DSBs, whereas Cpf1 cleavage produces cohesive ends. Third, Cpf1 recognizes thymidine-rich DNA sequences, such as the protospacer adjacent motifs (PAMs) at the 5' ends of target sequences (e.g., 5'-TTTN-3'). These features of Cpf1 broaden the range of CRISPR-endonuclease-editable genomic sites beyond the guanosine-rich sequences recognized by various Cas9 enzymes. In some embodiments, splitting the single guide RNA into two different nucleic acids (dual guide system) can provide improved flexibility and tunability. In some aspects, a dual guide system comprises (a) a targeter nucleic acid comprising: (i) a spacer sequence designed to hybridize with a target nucleotide sequence; and (ii) a targeter stem sequence, and (b) a modulator nucleic acid comprising a modulator stem sequence complementary to the targeter stem sequence, wherein the targeter nucleic acid and the modulator nucleic acid are separate nucleic acids, and wherein a complex comprising the targeter nucleic acid and the modulator nucleic acid is capable of activating a CRISPR Associated (Cas) nuclease that, in a naturally occurring system, is activated by a single crRNA in the absence of a tracrRNA (see for details the international patent application PCT/US202/0054050).

[0055] The term “recombination” and its grammatical equivalents as used herein can refer to a process of exchange of genetic information between two polynucleic acids. For the purposes of this disclosure, “homologous recombination” or “HR” can refer to a specialized form of such genetic exchange that can take place, for example, during repair of double-strand breaks. This process can require nucleotide sequence homology, for example, using a donor molecule to template repair of a target molecule (e.g., a molecule that experienced the double-strand break), and is sometimes known as non-crossover gene conversion or short tract gene conversion. Such transfer can also involve mismatch correction of heteroduplex DNA that forms between the broken target and the donor, and/or synthesis-dependent strand annealing, in which the donor can be used to resynthesize genetic information that can become part of the target, and/or related processes. Such specialized HR can often result in an alteration of the sequence of the target molecule such that part or all of the sequence of the donor polynucleotide can be incorporated into the target polynucleotide. In some cases, the terms “recombination arms” and “homology arms” can be used interchangeably.

[0056] As used herein, “operably linked” means that an element, such as an expression control sequence, is configured so as to perform its usual function upon a nucleotide sequence of interest. For example, a promoter operably linked to a nucleotide sequence of interest is capable

of effecting expression of the nucleotide sequence of interest. The expression control sequences need not be contiguous with the nucleotide sequence of interest, so long as they function to direct the expression thereof.

[0057] As used herein, the expression “percentage of identity” between two sequences, means the percentage of identical bases or amino acids between the two sequences to be compared, obtained with the best alignment of said sequences, this percentage being purely statistical and the differences between these two sequences being randomly spread over the two sequences. A base is considered complementary if it hybridizes under normal conditions. For example, a modified nucleobase can be aligned in a manner like the base whose hybridization pattern it mimics. As used herein, “best alignment” or “optimal alignment”, means the alignment for which the determined percentage of identity (see above) is the highest. Sequence comparison between two nucleic acid sequences (also referenced herein as nucleotide sequence or nucleobase sequence) is usually realized by comparing these sequences that have been previously aligned according to the best alignment. This comparison is realized on segments of comparison in order to identify and compared the local regions of similarity. The best sequence alignment to perform comparison can be realized, besides manually, by using the global homology algorithm developed by SMITH and WATERMAN (Ad. App. Math., vol.2, p:482, 1981), by using the local homology algorithm developed by NEEDLEMAN and WUNSCH (J. Mol. Biol, vol.48, p:443, 1970), by using the method of similarities developed by PEARSON and LIPMAN (Proc. Natl. Acad. Sci. USA, vol.85, p:2444, 1988), by using computer software using such algorithms (GAP, BESTFIT, BLAST P, BLAST N, FASTA, TFASTA in the Wisconsin Genetics software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, USA), by using the MUSCLE multiple alignment algorithms (Edgar, Robert C, Nucleic Acids Research, vol. 32, p: 1792, 2004). To get the best local alignment, one can preferably use BLAST software. The identity percentage between two sequences is determined by comparing these two sequences optimally aligned, the sequences being able to comprise additions or deletions with respect to the reference sequence in order to get the optimal alignment between these two sequences. The percentage of identity is calculated by determining the number of identical positions between these two sequences and dividing this number by the total number of compared positions, and by multiplying the result obtained by 100 to get the percentage of identity between these two sequences.

[0058] As used herein, “treatment”, or “treating” involves application of cells of the disclosure or a composition comprising the cells to a patient in need thereof with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease such as cancer, or any symptom of the disease (e.g., cancer or infectious disease). In particular, the terms “treat” or treatment” refers to reducing or alleviating at least one adverse clinical symptom associated with the disease. With reference to cancer treatment, the term “treat” or “treatment” also refers to slowing or reversing the progression of neoplastic uncontrolled cell multiplication, i.e. shrinking existing tumors and/or halting tumor growth. The term “treat” or “treatment” also refers to inducing apoptosis in cancer or tumor cells in the subject.

[0059] As used herein, “variant” means a sequence (polynucleotide or polypeptide) that has mutations (deletion, substitution or insertion) that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to a referenced sequence over its full length or over a region of at least 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, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, or 1100 nucleotides or amino acids. With respect to a polynucleotide sequence, variant also encompasses a polynucleotide that hybridizes under stringent conditions to the referenced sequence or complement thereof.

[0060] As used herein, a “vector” is any nucleic acid molecule for the transfer into or expression of a nucleic acid in a cell. The term “vector” includes both viral and nonviral (e.g., plasmid) nucleic acid molecules for introducing a nucleic acid into a cell *in vitro*, *ex vivo*, and/or *in vivo*. Vectors may include expression control sequences, restriction sites, and/or selectable markers. A “recombinant” vector refers to a vector that comprises one or more heterologous nucleotide sequences.

[0061] The term “transgene” and its grammatical equivalents as used herein can refer to a gene or genetic material that is transferred into an organism. For example, a transgene can be a stretch or segment of DNA containing a gene that is introduced into an organism. When a transgene is transferred into an organism, the organism is then referred to as a transgenic organism. A transgene can retain its ability to produce RNA or polypeptides (e.g., proteins) in a transgenic organism. A transgene can be composed of different nucleic acids, for example RNA or DNA. A transgene may encode for an engineered T cell receptor, for example a TCR transgene.

A transgene may comprise a TCR sequence. A transgene can comprise recombination arms. A transgene can comprise engineered sites.

Engineered immune cells

[0062] The cells are typically mammalian cells, or cell lines, e.g., mouse, rat, pig, non-human primate, or preferably human. Such cells include cells derived from the blood, bone marrow, lymph, or lymphoid organs (notably the thymus) and are preferably cells of the immune system (i.e., immune cells), such as cells of the innate or adaptive immunity, e.g., myeloid or lymphoid cells, including monocytes, macrophages, dendritic cells, or lymphocytes, typically T cells and/or NK cells.

[0063] Immune cells or progenitors thereof preferably also express one or more, or two or more, or three or more therapeutic proteins. In some embodiments, therapeutic proteins include antigen-specific receptors (CAR and/or TCR) as described herein, and optionally comprise one or more co-stimulatory receptors. Among the antigen-specific receptors according to the disclosure are recombinant modified T cell receptors (TCRs) and components thereof, as well as functional non-TCR antigen-specific receptors, such as chimeric antigen receptors (CAR).

[0064] Cells according to the disclosure may also be immune cell progenitors, such as lymphoid progenitors and more preferably T cell progenitors. Examples of T-cell progenitors include pluripotent stem cells (PSC), induced pluripotent stem cells (iPSCs) notably T-cell-derived induced pluripotent stem cells (TiPS) (see van der Stegen SJC, Lindenberg PL, Petrovic RM, et al. Generation of T-cell-receptor-negative CD8 $\alpha\beta$ -positive CAR T cells from T-cell-derived induced pluripotent stem cells. *Nat Biomed Eng.* 2022;6(11):1284-1297 and Themeli M, Kloss CC, Ciriello G, et al. Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy. *Nat Biotechnol.* 2013;31(10):928-933), hematopoietic stem cells (HSC), human embryonic stem cells (ESC), adipose-derived stem cells (ADSC), multipotent progenitor (MPP); lymphoid-primed multipotent progenitor (LMPP); common lymphoid progenitor (CLP); lymphoid progenitor (LP); thymus settling progenitor (TSP); or early thymic progenitor (ETP). Hematopoietic stem and progenitor cells can be obtained, for example, from cord blood, or from peripheral blood, e.g. peripheral blood-derived CD34⁺ cells after mobilization treatment with granulocyte-colony stimulating factor (G-CSF). T cell progenitors typically express a set of consensus markers including CD44, CD117, CD135, and/or Sca-1.

[0065] In some embodiments, the cells include one or more subsets of T cells or other cell types, such as whole T cell populations, CD4⁺ and/or CD8⁺ T cells, and subpopulations thereof, such as those defined by function, activation state, maturity, potential for differentiation, expansion, recirculation, localization, and/or persistence capacities, antigen-specificity, type of antigen-specific receptor, presence in a particular organ or compartment, marker or cytokine secretion profile, and/or degree of differentiation. In some embodiments, the cells include myeloid derived cells, such as dendritic cells, monocytes or macrophages.

[0066] Among the sub-types and subpopulations of T cells and/or of CD4⁺ and/or of CD8⁺ T cells are naive T (TN) cells, effector T cells (TEFF), memory T cells and sub-types thereof, such as stem cell memory T (TSCM), central memory T (TCM), effector memory T (TEM), or terminally differentiated effector memory T cells, tumor-infiltrating lymphocytes (TIL), immature T cells, mature T cells, helper T cells, cytotoxic T cells, mucosa-associated invariant T (MAIT) cells, naturally occurring and adaptive regulatory T (Treg) cells, helper T cells, such as TH1 cells, TH2 cells, TH3 cells, TH17 cells, TH9 cells, TH22 cells, follicular helper T cells, alpha/beta T cells, and delta/gamma T cells. Specifically contemplated herein are TEFF cells with stem/memory properties and higher reconstitution capacity due to the inhibition of SUV39H1, as well as TN cells, TSCM, TCM, TEM cells and combinations thereof.

[0067] In some embodiments, one or more of the T cell populations is enriched for, or depleted of, cells that are positive for or express high levels of one or more particular markers, such as surface markers, or that are negative for or express relatively low levels of one or more markers. In some cases, such markers are those that are absent or expressed at relatively low levels on certain populations of T cells (such as non-memory cells) but are present or expressed at relatively higher levels on certain other populations of T cells (such as memory cells). In one embodiment, the cells (such as the CD8⁺ cells or the T cells, e.g., CD3⁺ cells) are enriched for (i.e., positively selected for) cells that are positive or expressing high surface levels of CD117, CD135, CD45RO, CCR7, CD28, CD27, CD44, CD127, and/or CD62L and/or depleted of (e.g., negatively selected for) cells that are positive for or express high surface levels of CD45RA. In some embodiments, cells are enriched for or depleted of cells positive or expressing high surface levels of CD122, CD95, CD25, CD27, and/or IL7-Ra (CD127). In some examples, CD8⁺ T cells are enriched for cells positive for CD45RO (or negative for CD45RA) and for CD62L. The subset

of cells that are CCR7+, CD45RO+, CD27+, CD62L+ cells constitute a central memory cell subset.

[0068] For example, according to the disclosure, the cells can include a CD4+ T cell population and/or a CD8+ T cell sub-population, e.g., a sub-population enriched for central memory (TCM) cells. Alternatively, the cells can be other types of lymphocytes, including natural killer (NK) cells, mucosal associated invariant T (MAIT) cells, Innate Lymphoid Cells (ILCs) and B cells.

[0069] Cells include primary cells, isolated directly from a biological sample obtained from a subject, and optionally frozen. In some embodiments, the subject is in need of a cell therapy (adoptive cell therapy) and/or is the one who will receive the cell therapy. With reference to a subject to be treated with cell therapy, the cells may be allogeneic and/or autologous. In autologous immune cell therapy, immune cells are collected from the patient, modified as described herein, and returned to the patient. In allogeneic immune cell therapy, immune cells are collected from healthy donors, rather than the patient, modified as described herein, and administered to patients. Typically, these are HLA matched to reduce the likelihood of rejection by the host. The immune cells may also comprise modifications to reduce immunogenicity such as disruption or removal of HLA class I molecules, HLA-A locus, and/or Beta-2 microglobulin (B2M). For both autologous and non-autologous cells, the cells can optionally be cryopreserved until ready to be used for genetic manipulation and/or administration to a subject using methods well known in the art.

[0070] The samples include tissue samples, from tissues or organ, or fluid samples, such as blood, plasma, serum, cerebrospinal fluid, or synovial fluid. Samples may be taken directly from the subject, or may result from one or more processing steps, such as separation, centrifugation, genetic engineering (for example transduction with viral vector), washing, and/or incubation. Blood or blood-derived samples may be derived from an apheresis or a leukapheresis product. Exemplary samples include whole blood, peripheral blood mononuclear cells (PBMCs), leukocytes, bone marrow, thymus, tissue biopsy, tumor, leukemia, lymphoma, lymph node, gut associated lymphoid tissue, mucosa associated lymphoid tissue, spleen, other lymphoid tissues, myeloid derived cells, and/or cells derived therefrom.

[0071] SUV39H1 Human SUV39H1 methyltransferase is referenced as O43463 in UNIPROT and is encoded by the gene SUV39H1 located on chromosome x (gene ID: 6839 in NCBI). One exemplary human gene sequence is SEQ ID NO: 1, and one exemplary human protein sequence is SEQ ID NO: 2, but it is understood that polymorphisms or variants with different sequences exist in various subjects' genomes.

[0072] The term SUV39H1 according to the invention thus encompasses all mammalian variants of SUV39H1, and genes that encode a protein at least 75%, 80%, or typically 85%, 90%, or 95% identical to SEQ ID NO: 2 that has SUV39H1 activity (i.e., the methylation of Lys-9 of histone H3 by H3K9-histone methyltransferase). "Reduced expression of SUV39H1" as per the invention refers to a decrease of SUV39H1 expression of at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more as compared to normal levels in a control cell wherein the Suv39H1 gene has not been modified. By "non-functional" SUV39H1 protein it is herein intended a protein with a reduced activity (i.e., the methylation of Lys-9 of histone H3 by H3K9-histone methyltransferase), or a lack of detectable activity as described above, as compared to a control immune cell wherein the Suv39H1 gene has not been modified.

[0073] Cells according to the disclosure exhibit modulation, preferably complete inhibition, of SUV39H1 expression. Therefore, in some embodiments, the Suv39h1 gene is disrupted and/or inactivated. More particularly, the present disclosure provides an immune cell, wherein a therapeutic expression cassette transgene is integrated at a site within the genome of the cell such that expression of the transgene is under control of an endogenous or an exogenous promoter of said immune cell. Typically, the therapeutic expression cassette comprises at least an exogenous nucleic encoding at least one recombinant intracellular or cell-surface therapeutic protein. Cell surface recombinant proteins typically include chimeric antigen receptor and TCR (including modified TCR). In a preferred embodiment, the invention provides an immune cell, wherein a recombinant exogenous nucleic acid sequence encoding a therapeutic protein, notably an engineered antigen receptor (such as a CAR or a modified TCR) which is integrated at a site within the genome of the cell (typically expressed by the cell at the surface of the cell), and wherein the targeted integration of the nucleic acid encoding the therapeutic protein at a genomic locus reduces or prevents expression of Suv39H1.

[0074] In some specific embodiments, the present invention comprises the targeted integration of an expression cassette into a Suv39H1 gene site in an immune cell, preferably human T cells, wherein the transgene (exogenous nucleic acid) is encoding at least an engineered antigen receptor, a modified TCR and/or any other therapeutic protein. The transgene may be integrated in any one of exon 1 to 6 or in a non-coding regulatory region upstream of exon 1, or in any intronic region between exons 1 and 2, exons 2 and 3, exons 3 and 4, exons 4 and 5, and exons 5 and 6. In some cases, the transgene is integrated at an intronic locus of the Suv39H1 gene.

[0075] In certain embodiments, additional genomic locus can be targeted such as CD3S, CD3e, CD247, B2M, TRAC, TRBC1, TRBC2, TRGC1, HLA-E and/or TRGC2 loci.

[0076] The expression cassette can be constructed with an auxiliary molecule (e.g., a cytokine) in a single, multi cistronic expression cassette, in multiple expression cassettes of a single vector, or in multiple vectors. Examples of elements that create polycistronic expression cassette include, but is not limited to, various viral and non-viral Internal Ribosome Entry Sites (IRES, e.g., FGF-1 IRES, FGF-2 IRES, VEGF IRES, IGF-II IRES, NF- κ B IRES, RUNX1 IRES, p53 IRES, hepatitis A IRES, hepatitis C IRES, pestivirus IRES, aphthovirus IRES, picomavirus IRES, poliovirus IRES and encephalomyocarditis virus IRES) and cleavable linkers (e.g., 2 A peptides, e.g., P2A, T2A, E2A and F2A peptides). Combinations of retroviral vector and an appropriate packaging line are also suitable, where the capsid proteins will be functional for infecting human cells. Various amphotropic virus-producing cell lines are known, including, but not limited to, PA12 (Miller, et al. (1985) Mol. Cell. Biol. 5:431-437); PA317 (Miller, et al. (1986) Mol. Cell. Biol. 6:2895-2902); and CRIP (Danos, et al. (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464). Non- amphotropic particles are suitable too, e.g., particles pseudotyped with VSVG, RD114 or GALV envelope and any other known in the art.

[0077] In some embodiments, the expression cassette is “promoter-less” such that the expression of the transgene is under the control of an endogenous promoter. In some other embodiments the expression cassette includes a promoter that drives the expression of the transgene. Examples include CMV, EF-1a, hPGK and RPBSA. CAG promoters have been used to overexpress lncRNA. Yin et al Cell Stem Cell. 2015 May 7;16(5):504-16. Inducible promoters that are driven by signals from activated T cells include nuclear factor of activated T cells (NFAT) promoter. Other promoters for T cell expression of RNA include CIFT chimeric promoter

(containing portions of cytomegalovirus (CMV) enhancer, core interferon gamma (IFN- γ) promoter, JeT promoter (WO 2002/012514), and a T-lymphotropic virus long terminal repeat sequence (TLTR)), endogenous TRAC promoter or TRBC promoter. Inducible, constitutive, or tissue-specific promoters are contemplated.

5 **[0078]** In certain non-limiting embodiments, the expression of expression cassette integrated into a targeted genomic locus (e.g., SUV39H1 locus) is regulated by an endogenous transcription terminator of the genomic locus. In certain embodiments, the expression of an expression cassette integrated into a targeted genomic locus is regulated by a modified transcription terminator introduced to the genomic locus. Any targeted genome editing methods can be used to modify
10 the transcription terminator region of a targeted genomic locus, and thereby modifying the expression of an expression cassette in an immune cell of the present invention. In certain embodiments, the modification comprises replacement of an endogenous transcription terminator with an alternative transcription terminator, or insertion of an alternative transcription terminator to the transcription terminator region of a targeted genomic locus. In certain embodiments, the
15 alternative transcription terminator comprises a 3'UTR region or a ploy A region of a gene. In certain embodiments, the alternative transcription terminator is endogenous. In certain embodiments, the alternative transcription terminator is exogenous. In certain embodiments, alternative transcription terminators include, but are not limited to, a TK transcription terminator, a GCSF transcription terminator, a TCRA transcription terminator, an HBB transcription
20 terminator, a bovine growth hormone transcription terminator, an SV40 transcription terminator and a P2A element.

[0079] In a preferred embodiment, the recombinant cells can be used to enhance or provide an immune response against a desired target. In another embodiment, the recombinant cells can be used to inhibit an undesirable immune response. Preferably, the cells are derived from a human
25 (are of human origin prior to being made recombinant) (and human-derived cells are particularly preferred for administration to a human in the methods of treatment of the invention).

[0080] In some embodiments, the expression or the functionality of at least one further gene and/or protein is reduced or abolished in an immune cell of the present disclosure. The genes and proteins are typically selected among TRAC, TRBC, TRGC, TRDC, CD3 Delta, CD3 Epsilon,
30 CD3 Gamma, CD3 Zeta (CD247), B2M, CD4, CD8 alpha, CD8 beta, CTLA4, PD-1, TIM-3,

LAG3, TIGIT, CD28, CD25, CD69, CD95 (Fas), CD52, CD56, CD38, KLRG-1, SOCS1, CIITA, HLA genes (such as HLA-E) and NK specific genes (e.g, NKG2A, NKG2C, NKG2D, NKp46, CD 16, CD84, CD84, 2B4, and KIR-L) and protein encoded thereof. Inhibition of FAS, SOCS and/or a TRC gene are notably favored in allogenic applications. Inhibition of one or more of these genes can be achieved by contacting, or putting in contact, the cell with at least an agent inhibiting the expression and/or activity of SOCS-1, FAS, TCR or β 2m protein(s) and/or disrupting the FAS, β 2m SOCS-1 and/or TRC gene(s). Said agent can be selected from small molecule inhibitors; antibodies derivatives, aptamers, nucleic acid molecules that block transcription or translation, or gene editing agents targeting respectively SOCS1, FAS, TRC or B2N genes. Gene editing agents include CRISPR, TALEN, transposon-based, ZEN, meganuclease, or Mega-TAL agents.

[0081] Universal “off the shelf product” immune cells typically comprise modifications designed to reduce graft vs. host disease, such disruption or deletion of endogenous TCR. Because a single gene encodes the alpha chain (TRAC) rather than the two genes encoding the beta chain (TRBC), the TRAC locus is a common target for removing or disrupting endogenous TCR expression. Thus, in some embodiments, a further transgene is integrated at a TRC locus (such as the TRAC or the TRBC locus) of an immune cell such that the engineered immune cell expresses the transgene and has reduced or abolished expression of the endogenous TCR (a typical method is described in PCT/US2017/027601).

[0082] In some embodiments the expression of one or more additional proteins (including surface proteins) at multiple genomic loci (e.g., at least two, three, four, or five genomic loci), can be altered (i.e. reduced or abolished) notably using multiple simultaneous knockins (e.g. targeted insertion of a transgene at said genomic loci). The one or more proteins can be replaced with different exogenous (recombinant) intracellular or cell surface proteins, such as CAR, a TCR (including modified TCR such as HLA independent TCR) or any chimeric protein or receptor as defined herein.

In some embodiments, CRISPR editing agents can be used for multiplexed editing. In some cases, multiplexing can be performed by adding at least one guide RNA targeting the Suv39H1 locus, and at least one other guide targeting the one or more genomic loci, such as above described. In some embodiments, the gene disruption can be achieved as for the Suv39H1 disruption, by the

targeted insertion of an exogenous nucleic acid comprising a nucleic acid sequence coding for a therapeutic protein as herein described. Thus, in some embodiments, an immune cell defective for Suv39H1 comprises at least a nucleic acid coding for a therapeutic protein inserted at a Suv39H1 locus and at least a nucleic acid coding for a therapeutic protein (which can be the same or different) inserted at a second gene locus, which expression prevents the expression of a functional protein encoded by the said second gene.

[0083] In certain embodiments, a nucleic acid expression cassette is integrated at the Suv39H1 locus such that the Suv39H1 gene expression is reduced or suppressed. The nucleic acid expression cassette typically comprises at least one therapeutic transgene. The present application thus relates to immune cells expressing at least one recombinant therapeutic protein wherein said immune cell comprises at least one therapeutic transgene at a site within Suv39H1 gene region of the immune cell. A therapeutic transgene comprises a nucleotide (e.g., DNA or a modified form thereof) therapeutic nucleic sequence encoding a therapeutic protein. The therapeutic protein when expressed by the immune cell has use in treating a human or veterinary disease or disorder. The therapeutic protein can be a peptide or polypeptide.

[0084] Therapeutic nucleic acids include, but not limited to, those encoding a CAR, chimeric co-stimulatory receptor (CCR), modified TCR, TRC, cytokine, dominant negative, microenvironment modulator, antibody, biosensor, chimeric receptor ligand (CRL), chimeric immune receptor ligands (CIRL), soluble receptor, enzyme, ribozyme, genetic circuit, reporter, epigenetic modifier, transcriptional activator or repressor, non-coding RNA, or the like. It is understood that a transgene can encode, for example, a cDNA, a gene, miRNA or lncRNA, or the like. Additionally, the transgene can be a polycistronic message, i.e., arrayed cDNAs or arrayed miRNAs.

[0085] The cells of the disclosure with disrupted SUV39H1 expression include immune cells that express one or more, or two or more, or three or more antigen-specific receptors on their surface, and optionally one or more co-stimulatory receptors. Antigen-specific receptors include recombinant or modified T cell receptors (TCRs) and components thereof, and/or chimeric antigen receptors (CAR). For example, at least two CAR, at least two TCR, or at least one CAR with at least one TCR can be contemplated. The two or more antigen-specific receptors may bind the same or different antigen. In some embodiments, the two or more antigen-specific receptors

have different signaling domains. In some embodiments, the cell comprises an antigen-specific receptor with an activating signaling domain and an antigen-specific receptor with an inhibitory signaling domain. Typically, antigen-specific receptors bind the target antigen with a Kd binding affinity of about 10^{-6} M or less, about 10^{-7} M or less, about 10^{-8} M or less, about 10^{-9} M or less, about 10^{-10} M or less, or about 10^{-11} M or less (lower numbers indicating greater binding affinity).

Chimeric Antigen Receptors (CARs)

[0086] In some embodiments, the engineered antigen-specific receptors comprise chimeric antigen receptors (CARs), including activating or stimulatory CARs, costimulatory CARs (see WO2014/055668), and/or inhibitory CARs (iCARs, see Fedorov et al., Sci. Transl. Medicine, 5(215) (December 2013)).

[0087] Chimeric antigen receptors (CARs), (also known as Chimeric immunoreceptors, Chimeric T cell receptors, Artificial T cell receptors) are engineered antigen-specific receptors, which graft an arbitrary specificity onto an immune effector cell (T cell). Typically, these receptors are used to graft the specificity of a monoclonal antibody onto a T cell, with transfer of their coding sequence facilitated by retroviral vectors.

[0088] CARs generally include an extracellular antigen (or ligand) binding domain linked to one or more intracellular signaling components, in some aspects via linkers and/or transmembrane domain(s). Such molecules typically mimic or approximate a signal through a natural antigen receptor, a signal through such a receptor in combination with a costimulatory receptor, and/or a signal through a costimulatory receptor alone.

[0089] The CAR may include (a) an extracellular antigen-binding domain, (b) a transmembrane domain, (c) optionally a co-stimulatory domain, and (d) an intracellular signaling domain.

[0090] In some embodiments, the CAR is constructed with a specificity for a particular antigen (or marker or ligand), such as an antigen expressed in a particular cell type to be targeted by adoptive cell therapy, such as a cancer marker. The CAR typically includes in its extracellular portion one or more antigen binding molecules, such as one or more antigen-binding fragment, domain, or portion of an antibody, typically one or more antibody variable domains. For example, the extracellular antigen-binding domain may comprise a light chain variable domain or fragment thereof and/or a heavy chain variable domain or fragment thereof, typically as an scFv. In some

embodiments, the CAR comprises an antibody heavy chain variable domain or fragment thereof that specifically binds the antigen.

[0091] The moieties used to bind to antigen include three general categories, either single-chain antibody fragments (scFvs) derived from antibodies, Fab's selected from libraries, or natural ligands that engage their cognate receptor (for the first-generation CARs). Successful examples in each of these categories are notably reported in Sadelain M, Brentjens R, Riviere I. The basic principles of chimeric antigen receptor (CAR) design. *Cancer discovery*. 2013; 3(4):388-398 (see notably table 1) and are included in the present disclosure.

[0092] Antibodies include chimeric, humanized or human antibodies, and can be further affinity matured and selected as described above. Chimeric or humanized scFvs derived from rodent immunoglobulins (e.g. mice, rat) are commonly used, as they are easily derived from well-characterized monoclonal antibodies. Humanized antibodies contain rodent-sequence derived CDR regions. Typically the rodent CDRs are engrafted into a human framework, and some of the human framework residues may be back-mutated to the original rodent framework residue to preserve affinity, and/or one or a few of the CDR residues may be mutated to increase affinity. Fully human antibodies have no murine sequence and are typically produced via phage display technologies of human antibody libraries, or immunization of transgenic mice whose native immunoglobulin loci have been replaced with segments of human immunoglobulin loci. Variants of the antibodies can be produced that have one or more amino acid substitutions, insertions, or deletions in the native amino acid sequence, wherein the antibody retains or substantially retains its specific binding function. Conservative substitutions of amino acids are well known and described above. Further variants may also be produced that have improved affinity for the antigen.

[0093] In some embodiments, the modified TCR or CAR contains a fragment of an antibody or an antigen-binding fragment (e.g. single domain antibody, scFv, or variable heavy (VH) region and/or variable light (VL) region or 1, 2, or 3 CDRs of such VH and/or VL) that specifically recognizes an intracellular antigen, such as a tumor-associated antigen, presented on the cell surface as a MHC-peptide complex. Generally, a CAR containing an antibody or antigen-binding fragment that exhibits TCR-like specificity directed against peptide-MHC complexes also may be referred to as a TCR-like CAR.

[0094] The transmembrane domain in some embodiments is derived either from a natural or from a synthetic source. The transmembrane domain may be derived from the same receptor as the intracellular signaling domain, or a different receptor. Transmembrane regions include those derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD 134, CD137, CD154, ICOS or a GITR, or NKG2D, OX40, 2B4, DAP10, DAP12, or CD40. For T cells, CD8, CD28, CD3 epsilon may be preferred. For NK cells, NKG2D, DAP10, DAP12 may be preferred. In some embodiments, the transmembrane domain is derived from CD28, CD8 or CD3-zeta. In some instances, the transmembrane domain is selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

[0095] In some embodiments, a short oligo- or polypeptide linker, for example, a linker of between 2 and 10 amino acids in length, is present and forms a linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR.

[0096] The CAR generally includes at least one intracellular signaling component or components. First generation CARs typically had the intracellular domain from the CD3-zeta-chain, which is the primary transmitter of signals from endogenous TCRs. Second generation CARs typically further comprise intracellular signaling domains from various costimulatory protein receptors to the cytoplasmic tail of the CAR to provide additional signals to the T cell. Co-stimulatory domains include domains derived from human CD28, 4-1BB (CD137), ICOS, CD27, OX 40 (CD134), DAP10, DAP12, 2B4, CD40, FCER1G or GITR (AITR). For T cells, CD28, CD27, 4-1BB (CD137), ICOS may be preferred. For NK cells, DAP10, DAP12, 2B4 may be preferred. Combinations of two co-stimulatory domains are contemplated, e.g. CD28 and 4-1BB, or CD28 and OX40. Third generation CARs combine multiple signaling domains, such as CD3zeta-CD28-4-1BB or CD3zeta-CD28-OX40, to augment potency.

[0097] T cell activation is in some aspects described as being mediated by two classes of cytoplasmic signaling sequences: those that initiate antigen-dependent primary activation through the TCR (primary cytoplasmic signaling sequences), and those that act in an antigen-independent

manner to provide a secondary or co- stimulatory signal (secondary cytoplasmic signaling sequences). In some aspects, the CAR includes one or both of such signaling components.

[0098] In some aspects, the CAR includes a primary cytoplasmic signaling sequence that regulates primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary cytoplasmic signaling sequences that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine -based activation motifs or ITAMs. Examples of ITAM containing primary cytoplasmic signaling sequences include those derived from TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CDS, CD22, CD79a, CD79b, and CD66d. In some embodiments, cytoplasmic signaling molecule(s) in the CAR contain(s) a cytoplasmic signaling domain, portion thereof, or sequence derived from CD3 zeta. The CAR can also include a signaling domain and/or transmembrane portion of a costimulatory receptor, such as CD28, 4-1BB (CD137), ICOS, CD27, OX 40 (CD134), DAP10, DAP12, 2B4, CD40, FCER1G or GITR (AITR). In some aspects, the same CAR includes both the activating and costimulatory components; alternatively, the activating domain is provided by one CAR whereas the costimulatory component is provided by another CAR recognizing another antigen.

[0099] The intracellular signaling domain can be from an intracellular component of the TCR complex, such as a TCR CD3 chain that mediates T-cell activation and cytotoxicity, e.g., the CD3 zeta chain. Alternative intracellular signaling domains include FcεRIγ. The intracellular signaling domain may comprise a modified CD3 zeta polypeptide lacking one or two of its three immunoreceptor tyrosine-based activation motifs (ITAMs), wherein the ITAMs are ITAM1, ITAM2 and ITAM3 (numbered from the N- terminus to the C-terminus). The intracellular signaling region of CD3-zeta is residues 22-164. ITAM1 is located around amino acid residues 61-89, ITAM2 around amino acid residues 100-128, and ITAM3 around residues 131 -159. Thus, the modified CD3 zeta polypeptide may have any one of ITAM1, ITAM2, or ITAM3 inactivated, e.g. disrupted or deleted. Alternatively, the modified CD3 zeta polypeptide may have any two ITAMs inactivated, e.g. ITAM2 and ITAM3, or ITAM1 and ITAM2. Preferably, ITAM3 is inactivated, e.g. deleted. More preferably, ITAM2 and ITAM3 are inactivated, e.g. deleted, leaving ITAM1. For example, one modified CD3 zeta polypeptide retains only ITAM1 and the remaining CD3zeta domain is deleted (residues 90-164). As another example, ITAM1 is substituted with the amino acid sequence of ITAM3, and the remaining CD3zeta domain is

deleted (residues 90-164). See, for example, Bridgeman et al., Clin. Exp. Immunol. 175(2): 258-67 (2014); Zhao et al., J. Immunol. 183(9): 5563-74 (2009); Maus et al., WO-2018/132506; Sadelain et al., WO-2019/133969, Feucht et al., Nat Med. 25(1):82-88 (2019).

[00100] Thus, in some aspects, the antigen binding molecule is linked to one or more cell signaling modules. In some embodiments, cell signaling modules include CD3 transmembrane domain, CD3 intracellular signaling domains, and/or other CD transmembrane domains. The CAR can also further include a portion of one or more additional molecules such as Fc receptor g, CD8, CD4, CD25, or CD16.

[00101] In some embodiments, upon ligation of the CAR, the cytoplasmic domain or intracellular signaling domain of the CAR activates at least one of the normal effector functions or responses of the corresponding non-engineered immune cell (typically a T cell). For example, the CAR can induce a function of a T cell such as cytolytic activity or T-helper activity, secretion of cytokines or other factors.

[00102] The CAR or other antigen-specific receptor can also be an inhibitory CAR (e.g. iCAR) and includes intracellular components that dampen or suppress a response, such as an immune response. Examples of such intracellular signaling components are those found on immune checkpoint molecules, including PD-1, CTLA4, LAG3, BTLA, OX2R, TIM-3, TIGIT, LAIR-1, PGE2 receptors, or EP2/4 Adenosine receptors including A2AR. In some aspects, the engineered cell includes an inhibitory CAR including a signaling domain of or derived from such an inhibitory molecule, such that it serves to dampen the response of the cell. Such CARs are used, for example, to reduce the likelihood of off-target effects when the antigen recognized by the activating receptor, e.g. CAR, is also expressed, or may also be expressed, on the surface of normal cells.

TCRs

[00103] In some embodiments, the antigen-specific receptors include recombinant modified T cell receptors (TCRs) and/or TCRs cloned from naturally occurring T cells. Nucleic acid encoding the TCR can be obtained from a variety of sources, such as by polymerase chain reaction (PCR) amplification of naturally occurring TCR DNA sequences, followed by expression of antibody variable regions, followed by selecting for specific binding to antigen. In some embodiments, the TCR is obtained from T-cells isolated from a patient, or from cultured T-cell hybridomas. In some

embodiments, the TCR clone for a target antigen has been generated in transgenic mice engineered with human immune system genes (e.g., the human leukocyte antigen system, or HLA). See, e.g., tumor antigens (see, e.g., Parkhurst et al. (2009) Clin Cancer Res. 15:169-180 and Cohen et al. (2005) J Immunol. 175:5799-5808. In some embodiments, phage display is used to isolate TCRs against a target antigen (see, e.g., Varela-Rohena et al. (2008) Nat Med. 14:1390-1395 and Li (2005) Nat Biotechnol. 23:349-354.

[00104] A “T cell receptor” or “TCR” refers to a molecule that contains a variable alpha and beta chains (also known as TCR α and TCR β , respectively) or a variable gamma and delta chains (also known as TCR γ and TCR δ , respectively) and that is capable of specifically binding to an antigen peptide bound to a MHC receptor. In some embodiments, the antigen-binding domain of the TCR binds its target antigen with KD affinity of about 1×10^{-7} or less, about 5×10^{-8} or less, about 1×10^{-8} or less, about 5×10^{-9} or less, about 1×10^{-9} or less, about 5×10^{-10} or less, about 1×10^{-10} or less, about 5×10^{-11} or less, about 1×10^{-11} or less, about 5×10^{-12} or less, or about 1×10^{-12} or less (lower numbers indicating greater binding affinity). In some embodiments, the TCR is in the $\alpha\beta$ form. Typically, TCRs that exist in $\alpha\beta$ and $\gamma\delta$ forms are generally structurally similar, but T cells expressing them may have distinct anatomical locations or functions. A TCR can be found on the surface of a cell or in soluble form. Generally, a TCR is found on the surface of T cells (or T lymphocytes) where it is generally responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules. In some embodiments, a TCR also can contain a constant domain, a transmembrane domain and/or a short cytoplasmic tail (see, e.g., Janeway et al., Immunobiology: The Immune System in Health and Disease, 3rd Ed., Current Biology Publications, p. 4:33, 1997). For example, in some aspects, each chain of the TCR can possess one N-terminal immunoglobulin variable domain, one immunoglobulin constant domain, a transmembrane region, and a short cytoplasmic tail at the C-terminal end. In some embodiments, a TCR is associated with invariant proteins of the CD3 complex involved in mediating signal transduction. Unless otherwise stated, the term “TCR” should be understood to encompass functional TCR fragments thereof. The term also encompasses intact or full-length modified TCRs, including TCRs in the $\alpha\beta$ form or $\gamma\delta$ form.

[00105] Reference to a TCR includes any modified TCR or functional fragment thereof, such as an antigen-binding portion of a TCR that binds to a specific antigenic peptide bound in an MHC molecule, i.e. MHC-peptide complex. An “antigen binding portion” or “antigen-binding

fragment” of a TCR, which can be used interchangeably, refers to a molecule that contains a portion of the structural domains of a TCR, but that binds the antigen (e.g. MHC-peptide complex) to which the full TCR binds. In some cases, an antigen-binding portion contains the variable domains of a TCR, such as variable alpha chain and variable beta chain of a TCR, sufficient to form a binding site for binding to a specific MHC-peptide complex, such as generally where each chain contains three complementarity determining regions.

[00106] In some embodiments, the variable domains of the TCR chains associate to form loops, or complementarity determining regions (CDRs) analogous to immunoglobulins, which confer antigen recognition and determine peptide specificity by forming the binding site of the TCR molecule and determine peptide specificity. Typically, like immunoglobulins, the CDRs are separated by framework regions (FRs) (see, e.g., Jores et al., Proc. Nat'l. Acad. Sci. U.S.A. 87:9138, 1990; Chothia et al., EMBO J. 7:3745, 1988; see also Lefranc et al., Dev. Comp. Immunol. 27:55, 2003). In some embodiments, CDR3 is the main CDR responsible for recognizing processed antigen, although CDR1 of the alpha chain has also been shown to interact with the N-terminal part of the antigenic peptide, whereas CDR1 of the beta chain interacts with the C-terminal part of the peptide. CDR2 is thought to recognize the MHC molecule. In some embodiments, the variable region of the beta chain can contain a further hypervariability (HV4) region.

[00107] In some embodiments, the TCR chains contain a constant domain. For example, like immunoglobulins, the extracellular portion of TCR chains (e.g., α -chain, β -chain) can contain two immunoglobulin domains, a variable domain {e.g., $V\alpha$ or $V\beta$; typically amino acids 1 to 116 based on Kabat numbering Kabat et al., Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services, Public Health Service National Institutes of Health, 1991, 5th ed.) at the N-terminus, and one constant domain (e.g., α -chain constant domain or $C\alpha$ or TRAC, typically amino acids 117 to 259 based on Kabat, β -chain constant domain or $C\beta$ or TRBC, typically amino acids 117 to 295 based on Kabat) adjacent to the cell membrane. For example, in some cases, the extracellular portion of the TCR formed by the two chains contains two membrane-proximal constant domains, and two membrane-distal variable domains containing CDRs. The constant domain of the TCR domain contains short connecting sequences in which a cysteine residue forms a disulfide bond, making a link between the two chains. In some

embodiments, a TCR may have an additional cysteine residue in each of the α and β chains such that the TCR contains two disulfide bonds in the constant domains.

[00108] In some embodiments, the TCR chains can contain a transmembrane domain. In some embodiments, the transmembrane domain is positively charged. In some cases, the TCR chains contain a cytoplasmic tail. In some cases, the structure allows the TCR to associate with other molecules like CD3. For example, a TCR containing constant domains with a transmembrane region can anchor the protein in the cell membrane and associate with invariant subunits of the CD3 signaling apparatus or complex.

[00109] Generally, CD3 is a multi-protein complex that can possess three distinct chains (gamma (γ), delta (δ), and epsilon (ϵ)) and the zeta-chain. For example, in mammals the complex can contain a CD3gamma chain, a CD3delta chain, two CD3epsilon chains, and a homodimer of CD3zeta chains. The CD3gamma chains are highly related cell surface proteins of the immunoglobulin superfamily containing a single immunoglobulin domain. The transmembrane regions of the CD3gamma, CD3delta, and CD3epsilon chains are negatively charged, which is a characteristic that allows these chains to associate with the positively charged T cell receptor chains and play a role in propagating the signal from the TCR into the cell. The intracellular tails of the CD3gamma, CD3delta, and CD3epsilon chains each contain a single conserved motif known as an immunoreceptor tyrosine-based activation motif or ITAM, whereas each CD3 zeta chain has three ITAMs. Generally, ITAMs are involved in the signaling capacity of the TCR complex. The CD3gamma, delta, epsilon and zeta chains together form what is known as the T cell receptor complex.

[00110] The term “TCR” as used herein also includes (recombinant) TCRs modified to couple the antigen-binding utility of an antibody or fragment thereof to the endogenous TCR activation pathways. Typically, by employing an antibody or a fragment thereof as the antigen-binding domain, such modified TCRs can be used to target either peptide-MHC complexes or cell surface antigens by using either TCR-mimic (also named peptide-MHC restricted) antibodies or fragment(s) thereof or conventional antibodies (or fragment(s) thereof) respectively, such as e.g., a TCR modified to include a VH and/or VL of an antibody.

[00111] TCRs modified to couple the antigen-binding utility of an antibody (or fragment(s) thereof) to the endogenous TCR activation pathways have been broadly described for many years.

The method as herein described is compatible with many variations that have been described in the literature and/or in patent applications. Suitable examples include modified TCRs as described in Gross G, Gorochov G, Waks T, Eshhar Z. Generation of effector T cells expressing chimeric T cell receptor with antibody type-specificity. *Transplant Proc.* 1989;21(1 Pt 1):127-130 ; Yun CO, Nolan KF, Beecham EJ, Reisfeld RA, Junghans RP. Targeting of T lymphocytes to melanoma cells through chimeric anti-GD3 immunoglobulin T-cell receptors. *Neoplasia.* 2000;2(5):449-459 ; Xu Y, Yang Z, Horan LH, et al. A novel antibody-TCR (AbTCR) platform combines Fab-based antigen recognition with gamma/delta-TCR signaling to facilitate T-cell cytotoxicity with low cytokine release. *Cell Discov.* 2018;4:62 ; Baeuerle P. A., et al., Synthetic TRuC receptors engaging the complete T cell receptor for potent anti-tumor response. *Nat. Commun.* **10**, 1–12 (2019); Liu Y., Chimeric star receptors using TCR machinery mediate robust responses against solid tumors. *Sci. Transl. Med.* **13**, eabb5191 (2021); Mansilla-Soto J., et al., HLA-independent T cell receptors for targeting tumors with low antigen density. *Nat. Med.* **28**, 345–352 (2022), and He P, Liu H, Zimdahl B, et al. A novel antibody-TCR (AbTCR) T-cell therapy is safe and effective against CD19-positive relapsed/refractory B-cell lymphoma. *J Cancer Res Clin Oncol.* 2022;10.1007/s00432-022-04132-9 – Such modified TCRs have also been described in WO2016187349A1, WO2017059900A1, WO2017060300A1, WO2017070608A1, US2019359678A1, WO2020029774A1 and WO2021223707A1. For example, eTruC receptors fuse the scFv directly with the extracellular domain of CD3 ϵ (Baeuerle P. A., et al., Synthetic TRuC receptors engaging the complete T cell receptor for potent anti-tumor response. *Nat. Commun.* **10**, 1–12 (2019)), STARs and HIT receptors (Liu Y., Chimeric star receptors using TCR machinery mediate robust responses against solid tumors. *Sci. Transl. Med.* **13**, eabb5191 (2021); Mansilla-Soto J., et al., HLA-independent T cell receptors for targeting tumors with low antigen density. *Nat. Med.* **28**, 345–352 (2022)) are modified TCRs wherein the TCR α and β chain variable domains are replaced with the antibody variable domains (typically like scFv). Other teams have fused an antigen-binding domain from an antibody (notably a Fab fragment) with the C-terminal signaling domain of a $\gamma\delta$ TCR (Xu Y, Yang Z, Horan LH, et al. A novel antibody-TCR (AbTCR) platform combines Fab-based antigen recognition with gamma/delta-TCR signaling to facilitate T-cell cytotoxicity with low cytokine release. *Cell Discov.* 2018;4:62). Birtel et al., (Birtel M, Voss RH, Reinhard K, et al. A TCR-like CAR Promotes Sensitive Antigen Recognition and Controlled T-cell Expansion Upon mRNA

Vaccination. *Cancer Res Commun.* 2022;2(8):827-841) also developed a TCR-like CAR (TCAR) designed to closely mimic the dimeric TCR structure and physiologic signaling pathway similar to previous designs, wherein the scFv domains were fused to C α and C β . Identical variable domains were however arranged in tandem on either chain (VH-VH-C α /VL-VL-C β), see also
5 European patents EP3359563B1 and EP3596113B1.

[00112] Typically, in an HLA independent TCR, an antigen binding domain, such as one or more variable domains from an antibody (including fragments and derivatives thereof) are fused to one or more chains of the TCR. The variable domains of an antibody, fragments and derivatives thereof include in a non-limitative manner, (immunoglobulins such as IgG, single domain
10 antibodies (such as VHH or nanobodies), Fv, Fab, Fab', Fab'-SH, F(ab')₂; diabodies; linear antibodies; variable heavy chain (VH) regions, single-chain antibody molecules such as scFvs; and multispecific antibodies formed from antibody fragments).). In preferred embodiments, one or more antigen binding domain(s) replace(s) all or a portion thereof of an extracellular domain of one or more of the TCR chains. In some embodiments, the antigen binding domain is fused to
15 one or both of the TRAC or TRBC or a fragment or variant thereof, or to one or both of the gamma or delta constant chains. In some embodiments, a modified TCR comprises an antigen binding chain that comprises: (a) an antigen-binding domain as previously herein described that binds to an antigen in an HLA-independent manner, for example, an antigen-binding fragment of an immunoglobulin variable region; and (b) a constant domain that is capable of associating with
20 (and consequently activating) a CD3 ζ polypeptide. Because typically TCRs bind antigen in an HLA-dependent manner, the antigen-binding domain that binds in an HLA-independent manner must be heterologous. For example, an HLA independent TCR can comprise an extracellular antigen-binding domain that comprises one or two immunoglobulin variable region(s). Typical example of well suited antibodies and fragments thereof include, but are not limited to,
25 immunoglobulins such as IgG, single domain antibodies (such as VHH or nanobodies), Fv, Fab, Fab', Fab'-SH, F(ab')₂; diabodies; linear antibodies; variable heavy chain (VH) regions, single-chain antibody molecules such as scFvs; and multispecific antibodies formed from antibody fragments). The constant domain of the TCR is, for example, a native or modified TRAC polypeptide (including mutated and/or fragment thereof), or a native or modified TRBC
30 polypeptide (including mutated and/or fragment thereof). The constant domain of the TCR is, for example, a native TCR constant domain (from the alpha, beta, gamma and delta chains).

[0001] Examples of HLA independent (modified) TCRs are notably described in International Application No. WO 2019/157454. Such modified TCRs, designated HI-TCR or HIT-CAR herein, can comprise (a) a first antigen-binding chain of an antibody or a fragment thereof (for example comprising an antigen-binding fragment of a heavy chain variable region (VH)); and (b) a second antigen-binding chain of an antibody or a fragment thereof (for example comprising an antigen-binding fragment of a light chain variable region (VL)); wherein the first and second antigen-binding chains each comprise a native or variant TRAC (constant region) or fragment thereof, or a native or variant TRBC (constant region) or fragment thereof. In some embodiments, at least one of the TRAC polypeptide and the TRBC polypeptide is endogenous, therefore typically one or both of the endogenous TRAC and TRBC polypeptides is/are inactivated.

[0002] In some embodiments, the antigen-binding domain or fragment thereof comprises: (i) a heavy chain variable region (VH) of an antibody and/or (ii) a light chain variable region (VL) of an antibody. In some embodiments, the modified TCR is a gamma delta TCR and the constant domains are the constant gamma and delta chains.

[00113] In some embodiments of the present invention, a recombinant HLA independent TCR is inserted at a SUV39H1 locus. Typically such HLA independent TCR comprises exogenous TRAC and TRBC sequences (or exogenous constant gamma and delta sequences). In some designs, the HI-TCR therefore comprises (a) a chimeric TCR alpha chain comprising a VH or fragment thereof fused to a native or variant TRAC or fragment thereof, optionally in which amino acids of the VH (or the TRAC) are removed, optionally in which one to three amino acids of the VH (or the TRAC) are removed, and (b) a chimeric TCR beta chain comprising a VL or fragment thereof fused to a native or variant TRBC or fragment thereof, optionally in which amino acids of the VL (or the TRBC) are removed, optionally in which one to three amino acids of the VL (or the TRBC) are removed. In other designs, the HI-TCR comprises (a) a chimeric TCR alpha chain comprising a VL or fragment thereof fused to a native or variant TRAC or fragment thereof, optionally in which amino acids of the VL (or TRAC) are removed, and (b) a chimeric TCR beta chain comprising a VH or fragment thereof fused to a native or variant TRBC or fragment thereof, optionally in which amino acids of the VH (or TRBC) are removed. In yet other designs, the HI-TCR comprises just an antigen binding domain or fragment thereof (such as an scFv, a VHH, a VH or a VL) fused to a native or variant TRAC or fragment thereof, or

fused to a native or variant TRBC or fragment thereof, optionally in which amino acids of the VH (or TRAC or TRBC) are removed. HI-TCR (HIT-CAR) are described in Int'l Pat. Pub. No. WO 2019/157454, incorporated by reference herein in its entirety.

5 [00114] Other HLA independent TCRs are disclosed in Int'l Pat. Pub. No. WO 2018/067993, incorporated herein by reference in its entirety, as well as in Baeuerle, et al. "*Synthetic TRuC receptors engaging the complete T cell receptor for potent anti-tumor response*". Nat Commun 10, 2087 (2019). For example, any one or more, or two or more, of the alpha, beta, gamma, delta or epsilon chains (e.g. intracellular and optionally transmembrane domains thereof) may be fused to an antibody variable region or a fragment or derivatives thereof as above exemplified (e.g., 10 VH and/or VL, scFv, or VHH).

[00115] In some embodiments, a nucleic acid encoding the heterologous antigen-binding domain (e.g., scFv, VHH, VH or variant or fragment thereof, or VL or variant or fragment thereof as previously exemplified) is inserted into the endogenous TRAC locus and/or TRBC locus of the immune cell. Optionally, the nucleic acid encoding the chimeric TCR alpha (or beta) chain 15 is operatively linked to an endogenous promoter of the T-cell receptor such that its expression is under control of the endogenous promoter. The insertion of the nucleic acid sequence can also inactivate or disrupt the endogenous expression of a TCR comprising a native TCR alpha chain and/or a native TCR beta chain. The insertion of the nucleic acid sequence may reduce endogenous TCR expression by at least about 75%, 80%, 85%, 90% or 95%.

20 [00116] In certain embodiments, the extracellular antigen-binding domain comprises a heavy chain variable region (V_H) and/or a light chain variable region (V_L) of an antibody, wherein the V_H or the V_L is capable of dimerizing with another extracellular antigen binding domain comprising a VL or a VH (e.g., forming a fragment variable (Fv)). In certain embodiments, the Fv is a human Fv. In certain embodiments, the Fv is a humanized Fv. In certain embodiments, 25 the Fv is a murine Fv. In certain embodiments, the Fv is identified by screening a Fv phage library with an antigen-Fc fusion protein. Additional extracellular antigen-binding domains targeting an interested antigen can be obtained by sequencing an existing scFv or a Fab region of an existing antibody targeting the same antigen.

[0003] In a recombinant TCR as per the present invention, the TCR sequence can be of human 30 or murine origin. The TRAC and/or TRBC sequence can exhibit one or more mutations as

compared to the endogenous human sequence, or can be a fragment thereof. In some embodiments the modified TCR as per the present invention includes a modified TRAC and or TRBC sequence as compared to the endogenous TRAC or TRBC constant domain sequence. Preferably the TRAC and/or the TRBC sequence of the modified TCR comprises at least a base deletion or a substitution as compared to the endogenous TRAC and/or TRBC sequence, wherein the base deletion or the substitution reduces binding of a GMP-grade anti (human) antibody directed against a TRAC or TRBC sequence to the mutated TRAC or TRBC sequence, optionally wherein the modified TRAC and/or TRBC sequence has at least 95%, notably at least 96 %, 97 %, 98 % or 99 % identity with the corresponding endogenous TRAC and/or TRBC sequence. In some embodiments of a modified TCR, the TRBC sequence is modified such that binding to an anti-TCR α/β antibody, which recognizes a common determinant of the TCR α/β -CD3 complex, such as the BW242/412 antibody, is abolished.

[00117] Unlike chimeric antigen receptors, which typically themselves comprise an intracellular signaling domain, the HI-TCR does not directly produce an activating signal; instead, the antigen-binding chain associates with and consequently activates a CD3zeta polypeptide. The immune cells comprising the recombinant TCR provide superior activity when the antigen has a low density on the cell surface of less than about 10,000 molecules per cell, e.g. less than about 5,000, 4,000, 3,000, 2,000, 1,000, 500, 250 or 100 molecules per cell. In some embodiments, the antigen is expressed at low density by the target cell, e.g., less than about 6,000 molecules of the target antigen per cell. In some embodiments, the antigen is expressed at a density of less than about 5,000 molecules, less than about 4,000 molecules, less than about 3,000 molecules, less than about 2,000 molecules, less than about 1,000 molecules, or less than about 500 molecules of the target antigen per cell. In some embodiments, the antigen is expressed at a density of less than about 2,000 molecules, such as e.g., less than about 1,800 molecules, less than about 1,600 molecules, less than about 1,400 molecules, less than about 1,200 molecules, less than about 1,000 molecules, less than about 800 molecules, less than about 600 molecules, less than about 400 molecules, less than about 200 molecules, or less than about 100 molecules of the target antigen per cell. In some embodiments, the antigen is expressed at a density of less than about 1,000 molecules, such as e.g., less than about 900 molecules, less than about 800 molecules, less than about 700 molecules, less than about 600 molecules, less than about 500 molecules, less than about 400 molecules, less than about 300 molecules, less than about 200 molecules, or less than

about 100 molecules of the target antigen per cell. In some embodiments, the antigen is expressed at a density ranging from about 5,000 to about 100 molecules of the target antigen per cell, such as e.g., from about 5,000 to about 1,000 molecules, from about 4,000 to about 2,000 molecules, from about 3,000 to about 2,000 molecules, from about 4,000 to about 3,000 molecules, from about 3,000 to about 1,000 molecules, from about 2,000 to about 1,000 molecules, from about 1,000 to about 500 molecules, from about 500 to about 100 molecules of the target antigen per cell. In some embodiments, the recombinant TCR T cell therapy targets an antigen that is expressed at low density compared to a density in a wild-type cell. Typically, the antigen is expressed at a density of at least 10, at least 20, at least 30, at least 40, at least 50 molecules per cell. Typically it is expressed at a cell density ranging from 10, notably from 20, 30, 40 or 50 molecules per cell to less than 500, notably less than 300, 250, 200, 150 or 100 molecules per cell.

[00118] The CD3zeta polypeptide optionally comprises an intracellular domain of a co-stimulatory molecule or a fragment thereof. Alternatively, the antigen binding domain optionally comprises a co-stimulatory domain that is capable of stimulating an immunoresponsive cell upon the binding of the antigen binding chain to the antigen. Example co-stimulatory domains include stimulatory domains, or fragments or variants thereof, from CD28, 4-1BB (CD137), ICOS, CD27, OX 40 (CD134), DAP10, DAP12, 2B4, CD40, FCER1G or GITR (AITR). For T cells, CD28, CD27, 4-1BB (CD137), ICOS may be preferred. For NK cells, DAP10, DAP12, 2B4 may be preferred. Combinations of two co-stimulatory domains are contemplated, e.g. CD28 and 4-1BB, or CD28 and OX40.

[00119] The foregoing modified immune cell expressing an antigen-specific receptor, e.g. a modified (recombinant) TCR, preferably comprises one or more further features as described herein: inactivation of the SUV39H1 gene, and/or inactivation of one or two ITAM domains of the CD3zeta intracellular signaling region of the antigen-specific receptor, and/or inactivation of the endogenous TCR receptor (through knock out or knock down of one or both of the TRAC and TRBC chain, preferably the TRAC chain) and/or addition of a co-stimulatory receptor, or combinations of one, two, three or all of such features. In some embodiments, the recombinant TCR chains contain a constant domain. For example, like immunoglobulins, the extracellular portion of TCR chains (e.g., α -chain, β -chain) can contain two immunoglobulin domains, a variable domain (e.g., $V\alpha$ or $V\beta$; typically amino acids 1 to 116 based on Kabat numbering Kabat

et al., "Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services, Public Health Service National Institutes of Health, 1991, 5th ed.) at the N-terminus, and one constant domain (e.g., α -chain constant domain or C α , typically amino acids 117 to 259 based on Kabat, or β -chain constant domain or C β , typically amino acids 117 to 295 based on Kabat) adjacent to the cell membrane. For example, in some cases, the extracellular portion of the recombinant TCR formed by the two chains contains two membrane-proximal constant domains, and two membrane-distal variable domains containing CDRs. The constant domain of the TCR domain contains short connecting sequences in which a cysteine residue forms a disulfide bond, making a link between the two chains. In some embodiments, a TCR may have an additional cysteine residue in each of the α and β chains such that the TCR contains two disulfide bonds in the constant domains.

[00120] In some embodiments, the TCR chains can contain a transmembrane domain. In some embodiments, the transmembrane domain is positively charged. In some cases, the (recombinant or modified) TCR chains contain a cytoplasmic tail. In some cases, the structure allows the (recombinant) TCR to associate with other molecules like CD3. For example, a (recombinant) TCR containing constant domains with a transmembrane region can anchor the protein in the cell membrane and associate with invariant subunits of the CD3 signaling apparatus or complex.

[00121] Other examples of antigen-specific receptors, including CARs and recombinant modified TCRs, as well as methods for engineering and introducing the receptors into cells, include those described, for example, in international patent application publication numbers WO-2000/014257, WO-2013/126726, WO-2012/129514, WO-2014/031687, WO-2013/166321, WO-2013/071154, WO-2013/123061 U.S. patent application publication numbers US-2002131960, US-2013287748, US-20130149337, U.S. Patent Nos.: 6,451,995, 7,446,190, 8,252,592, 8,339,645, 8,398,282, 7,446,179, 6,410,319, 7,070,995, 7,265,209, 7,354,762, 7,446,191, 8,324,353, and 8,479,118, and European patent application number EP2537416, and/or those described by Sadelain et al., Cancer Discov. 2013 April; 3(4): 388-398; Davila et al. (2013) PLoS ONE 8(4): e61338; Turtle et al., Curr. Opin. Immunol., 2012 October; 24(5): 633-39; Wu et al., Cancer, 2012 March 18(2): 160-75. In some aspects, the antigen-specific receptors include a CAR as described in U.S. Patent No.: 7,446,190, and those described in International Patent Application Publication No.: WO-2014/055668 A1.

Co-stimulatory ligands and receptors

[00122] The cells of the disclosure with modified SUV39H1 expression may further comprise at least one or at least two exogenous co-stimulatory ligands.

[00123] In certain embodiments, the immune cell comprises an exogenous or a recombinant (e.g., the cell is transduced with at least one) co-stimulatory ligand. In certain embodiments, the immune cell co-expresses a CAR or an exogenous TCR (including modified TCR) and the at least one exogenous co-stimulatory ligand. The interaction between the CAR or the modified TCR and at least one exogenous co-stimulatory ligand provides a non-antigen-specific signal important for full activation of an immunoresponsive cell (e.g, T cell). Co-stimulatory ligands include, but are not limited to, members of the tumor necrosis factor (TNF) superfamily, and immunoglobulin (Ig) superfamily ligands. TNF is a cytokine involved in systemic inflammation and stimulates the acute phase reaction. Its primary role is in the regulation of immune cells. Members of TNF superfamily share a number of common features. The majority of TNF superfamily members are synthesized as type II transmembrane proteins (extracellular C-terminus) containing a short cytoplasmic segment and a relatively long extracellular region. TNF superfamily members include, but are not limited to, nerve growth factor (NGF), CD40L (CD40L)/CD154, CD137L/4-1BBL, TNF- α , CD134L/OX40L/CD252, CD27L/CD70, Fas ligand (FasL), CD30L/CD153, tumor necrosis factor beta (TNFP)/lymphotoxin- α (LT α), lymphotoxin- β (TT β), CD257/B cell-activating factor (BAFF)/Blys/THANK/Tall-1, glucocorticoid-induced TNF Receptor ligand (GITRL), and TNF-related apoptosis-inducing ligand (TRAIL), LIGHT (TNFSF14). The immunoglobulin (Ig) superfamily is a large group of cell surface and soluble proteins that are involved in the recognition, binding, or adhesion processes of cells. These proteins share structural features with immunoglobulins— they possess an immunoglobulin domain (fold). Immunoglobulin superfamily ligands include, but are not limited to, CD80 and CD86, both ligands for CD28. In certain embodiments, the at least one co-stimulatory ligand is selected from the group consisting of 4-1BBL, CD275, CD80, CD86, CD70, OX40L, CD48, TNFRSF14, and combinations thereof. In certain embodiments, the immunoresponsive cell comprises or consists of one exogenous or recombinant co-stimulatory ligand. In certain embodiments, the one exogenous or recombinant co-stimulatory ligand is 4-1BBL or CD80. In certain embodiments, the one exogenous or recombinant co-stimulatory ligand is 4-1BBL. In certain embodiments, the immunoresponsive cell comprises or consists of two exogenous or recombinant co-stimulatory

ligands. In certain embodiments, the two exogenous or recombinant co-stimulatory ligands are 4-1BBL and CD80.

[00124] In some embodiments, the co-stimulatory ligand is CD80 and/or 4-1BBL. Example co-stimulatory molecules are CD28, 4-1BB, OX40, ICOS, DAP-10, CD27, CD40, NKG2D, CD2, or any combination thereof. In some embodiments, the chimeric receptor or molecule comprises a first co-stimulatory molecule that is 4-1BB and a second co-stimulatory molecule that is CD28. In some embodiments, the cell comprises a co-stimulatory molecule (i.e. a fusion polypeptide) comprising an extracellular domain and a transmembrane domain of a co-stimulatory ligand, and an intracellular domain of a first co-stimulatory molecule. In some embodiments, the co-stimulatory ligand can be selected from the group consisting of a tumor necrosis factor (TNF) family member, an immunoglobulin (Ig) superfamily member, and combinations thereof. Typically, the TNF family member can be selected from the group consisting of 4-1BBL, OX40L, CD70, GITRL, CD40L, and combination thereof. The Ig superfamily member can also be selected from the group consisting of (typically human) CD80, CD86, ICOSLG, and combinations thereof. The first co-stimulatory molecule can for example be selected from the group consisting of CD28, 4-1BB, OX40, ICOS, DAP-10, CD27, CD40, NKG2D, CD2, and combinations thereof. Typically, the co-stimulatory ligand is CD80 and the first co-stimulatory molecule can be selected from the group consisting of (typically human) CD28, 4-1BB, OX40, ICOS, DAP-10, CD27, CD40, NKG2D, CD2, and combinations thereof. In some embodiments, the co-stimulatory receptor or ligand is a fusion polypeptide comprising the extracellular domain of human CD80, transmembrane domain of human CD80, and an intracellular human 4-1BB domain.

[00125] In certain embodiments, the immunoresponsive cell can comprise or be transduced with at least one chimeric co-stimulatory receptor (CCR). As used herein, the term “chimeric co-stimulatory receptor” or “CCR” refers to a chimeric receptor that binds to an antigen, and, upon its binding to the antigen, provides a co-stimulatory signal to a cell (e.g., a T cell) comprising the CCR, but does not alone provide an activation signal to the cell. CCR is described in Krause, et al., J. Exp. Med. (1998);188(4):619-626, and US20020018783, which is incorporated by reference in its entirety. CCRs mimic co-stimulatory signals, but unlike, CARs, do not provide a T-cell activation signal, e.g., CCRs lack a E03z polypeptide. CCRs provide co-stimulation, e.g., a CD28-like signal, in the absence of the natural co-stimulatory ligand on the antigen-presenting

cell. A combinatorial antigen recognition, i.e., use of a CCR in combination with a CAR, can augment T-cell reactivity against the dual-antigen expressing T cells, thereby improving selective tumor targeting. See WO2014/055668, which is incorporated by reference in its entirety. Kloss et al., describe a strategy that integrates combinatorial antigen recognition, split signaling, and, critically, balanced strength of T-cell activation and co stimulation to generate T cells that eliminate target cells that express a combination of antigens while sparing cells that express each antigen individually (Kloss et al., Nature Biotechnology (2013);3 1(1):71-75, the content of which is incorporated by reference in its entirety). With this approach, T-cell activation requires CAR-mediated recognition of one antigen, whereas co- stimulation is independently mediated by a CCR specific for a second antigen. To achieve tumor selectivity, the combinatorial antigen recognition approach diminishes the efficiency of T-cell activation to a level where it is ineffective without rescue provided by simultaneous CCR recognition of the second antigen. In certain embodiments, the CCR comprises an extracellular antigen-binding domain that binds to a second antigen, a transmembrane domain, and a co-stimulatory signaling region that comprises at least one co-stimulatory molecule. In certain embodiments, the CCR does not alone deliver an activation signal to the cell. Non limiting examples of co-stimulatory molecules include CD28, 4-1BB, OX40, ICOS, DAP- 10 and any combination thereof. In certain embodiments, the co-stimulatory signaling region of the CCR comprises one co-stimulatory signaling molecule. In certain embodiments, the one co-stimulatory signaling molecule is CD28. In certain embodiments, the one co-stimulatory signaling molecule is 4-1BB. In certain embodiments, the co-stimulatory signaling region of the CCR comprises two co stimulatory signaling molecules. In certain embodiments, the two co-stimulatory signaling molecules are CD28 and 4-1BB.

[00126] A second antigen is selected so that expression of both the first antigen and the second antigen is restricted to the targeted cells (e.g., cancerous tissue or cancerous cells). Similar to a CAR, the extracellular antigen-binding domain can be a scFv, a Fab, a F(ab)₂, or a fusion protein with a heterologous sequence to form the extracellular antigen-binding domain. In certain embodiments, the CCR is co-expressed with a CAR or a modified TCR binding to an antigen that is different from the antigen to which the CCR binds, e.g., the CAR or the modified TCR binds to a first antigen and the CCR binds to a second antigen.

[00127] Example co-stimulatory ligands, molecules and receptors (or fusion polypeptides) are described in Int'l Pat. Pub. No. WO-2021/016174, incorporated by reference herein in its entirety.

Illustrative costimulatory receptors or molecules include molecules of SEQ ID NO: 32-33 and 53-54.

[00128] The cells of the disclosure with modulated SUV39H1 expression may also comprise T-cell specific engagers, such as BiTEs, or bispecific antibodies that bind not only the desired antigen but also an activating T-cell antigen such as CD3 epsilon. In some embodiments, the BiTe comprises an antigen-binding domain, e.g. scFv, linked to a T-cell recognizing domain, e.g., heavy variable domain and/or light variable domain of an anti-CD3 antibody.

Antigens

[00129] Antigens include antigens associated with diseases or disorders, including proliferative, neoplastic, and malignant diseases and disorders, more particularly cancers. Infectious diseases and autoimmune, inflammatory or allergic diseases are also contemplated.

[00130] The cancer may be a solid cancer (solid tumor) or a “liquid tumor” such as cancers affecting the blood, bone marrow and lymphoid system, also known as tumors of the hematopoietic and lymphoid tissues, which notably include leukemia and lymphoma. Liquid tumors include for example acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL), (including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma (NHL) Central nervous system lymphoma (CNSL), adenoma, squamous cell carcinoma, laryngeal carcinoma, gallbladder and bile duct cancers, cancers of the retina such as retinoblastoma).

[00131] Solid cancers (also called solid tumors) notably include cancer affecting an organ, optionally colon, rectum, skin, endometrium, lung (including non-small cell lung carcinoma), uterus, bones (such as Osteosarcoma, Chondrosarcomas, Ewing's sarcoma, Fibrosarcomas, Giant cell tumors, Adamantinomas, and Chordomas), liver, kidney, esophagus, stomach, bladder, pancreas, cervix, brain (such as Meningiomas, Glioblastomas, Lower-Grade Astrocytomas, Oligodendrocytomas, Pituitary Tumors, Schwannomas, and Metastatic brain cancers), ovary, breast (including triple negative breast cancer), head and neck region, testis, prostate or the thyroid gland.

[00132] Cancers include cancers affecting the blood, bone marrow and lymphoid system as described above. In some embodiments, the cancer is, or is associated, with multiple myeloma.

Antigens associated with multiple myeloma include CD38, CD138, and/or CS-1. Other exemplary multiple myeloma antigens include CD56, TIM-3, CD33, CD123, and/or CD44.

[00133] Diseases also encompass infectious diseases or conditions, such as, but not limited to, viral, retroviral, bacterial, protozoal or parasitic, infections, or viral infections caused by, e.g., human immunodeficiency virus (HIV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus, BK polyomavirus, hepatitis virus, such as hepatitis B, hepatitis C, hepatitis D, hepatitis E.

[00134] In some embodiments the extracellular antigen-binding domain binds to any of the tumor neoantigenic peptides disclosed in Int'l Pat. Pub. No. WO 2021/043804, incorporated by reference herein in its entirety. For example, the antigen-binding domain binds to any of the peptides of F or to a neoantigenic peptide comprising at least 8, 9, 10, 11 or 12 amino acids that is encoded by a part of an open reading frame (ORF) of any of the fusion transcript sequences of any one of SEQ ID NO: 118-17492 of WO 2021/043804.

[00135] Diseases also encompass autoimmune or inflammatory diseases or conditions, such as arthritis, e.g., rheumatoid arthritis (RA), Type I diabetes, systemic lupus erythematosus (SLE), inflammatory bowel disease, psoriasis, scleroderma, autoimmune thyroid disease, Grave's disease, Crohn's disease multiple sclerosis, asthma, and/or diseases or conditions associated with transplant. In such circumstances, a T-regulatory cell may be the cell in which SUV39H1 is inhibited.

[00136] In some embodiments, the antigen is a polypeptide. In some embodiments, it is a carbohydrate or other molecule. In some embodiments, the antigen is selectively expressed or overexpressed on cells of the disease or condition, e.g., the tumor or pathogenic cells, as compared to normal or non-targeted cells or tissues. In other embodiments, the antigen is expressed on normal cells and/or is expressed on the engineered cells. In some such embodiments, a multi-targeting and/or gene disruption approach as provided herein is used to improve specificity and/or efficacy.

[00137] In some embodiments, the antigen is a universal tumor antigen. The term "universal tumor antigen" refers to an immunogenic molecule, such as a protein, that is, generally, expressed at a higher level in tumor cells than in non-tumor cells and also is expressed in tumors of different origins. In some embodiments, the universal tumor antigen is expressed in more than 30%, 40%,

50%, 60%, 70%, 75%, 80%, 85%, 90% or more of human cancers. In some embodiments, the universal tumor antigen is expressed in at least three, at least four, at least five, at least six, at least seven, at least eight or more different types of tumors. In some cases, the universal tumor antigen may be expressed in non-tumor cells, such as normal cells, but at lower levels than it is expressed in tumor cells. In some cases, the universal tumor antigen is not expressed at all in non-tumor cells, such as not expressed in normal cells. Exemplary universal tumor antigens include, for example, human telomerase reverse transcriptase (hTERT), survivin, mouse double minute 2 homolog (MDM2), cytochrome P450 1 B1 (CYP1 B), HER2/neu, p95HER2, Wilms tumor gene 1 (WT1), livin, alphafetoprotein (AFP), carcinoembryonic antigen (CEA), mucin 16 (MUC16), MUC1, prostate-specific membrane antigen (PSMA), p53 or cyclin (D1). Peptide epitopes of tumor antigens, including universal tumor antigens, are known in the art and, in some aspects, can be used to generate MHC-restricted antigen-specific receptors, such as TCRs or TCR-like CARs (see e.g. published PCT application No. WO-2011/009173 or WO-2012/135854 and published U.S. application No. US-20140065708).

[00138] In some embodiments, the cancer is, or is associated, with overexpression of HER2 or p95HER2. p95HER2 is a constitutively active C-terminal fragment of HER2 that is produced by an alternative initiation of translation at methionine 611 of the transcript encoding the full-length HER2 receptor. HER2 or p95HER2 has been reported to be overexpressed in breast cancer, as well as gastric (stomach) cancer, gastroesophageal cancer, esophageal cancer, ovarian cancer, uterine endometrial cancer, cervix cancer, colon cancer, bladder cancer, lung cancer, and head and neck cancers. Patients with cancers that express the p95HER2 fragment have a greater probability of developing metastasis and a worse prognosis than those patients who mainly express the complete form of HER2. Saez et al., *Clinical Cancer Research*, 12:424-431 (2006).

[00139] HER2 or p95HER2 has been reported to be overexpressed in breast cancer, as well as gastric (stomach) cancer, gastroesophageal cancer, esophageal cancer, ovarian cancer, uterine endometrial cancer, cervix cancer, colon cancer, bladder cancer, lung cancer, and head and neck cancers. Patients with cancers that express the p95HER2 fragment have a greater probability of developing metastasis and a worse prognosis than those patients who mainly express the complete form of HER2. Saez et al., *Clinical Cancer Research*, 12:424-431 (2006). Antibodies that can specifically bind p95HER2 compared to HER2 (i.e., bind p95HER2 but do not bind significantly to full length HER2 receptor) are disclosed in Sperinde et al., *Clin. Cancer Res.* 16, 4226–4235

(2010) and U.S. Patent Pub. No. 2013/0316380, incorporated by reference herein in their entireties. Hybridomas that produce monoclonal antibodies that can specifically bind p95HER2 compared to HER2 are disclosed in Int'l. Patent Pub. No. WO/2010/000565, and in Parra-Palau et al., *Cancer Res.* 70, 8537–8546 (2010). An example CAR binds the epitope PIWKFPD of p95HER2 with a binding affinity K_D of 10^{-7} M or less, 10^{-8} M or less, 10^{-9} M or less or 10^{-10} M or less. In some embodiments, a CAR or a modified TCR (e.g. Hi-TCR) as herein described comprises VH/VL sequences as described in WO2021239965. Rius Ruiz et al., *Sci. Transl. Med.* 10, eaat1445 (2018) and U.S. Patent Pub. No. 2018/0118849, incorporated by reference herein in their entireties, describe a T-cell bispecific antibody that specifically binds to the epitope PIWKFPD of p95HER2 and to the CD3 epsilon chain of the TCR. The antibody designated p95HER2-TCB consists of an asymmetric two-armed immunoglobulin G1 (IgG1) that binds monovalently to CD3 epsilon and bivalently to p95HER2. The bispecific antibody has monovalent low affinity for CD3 epsilon of about 70 to 100 nM which reduces the chances of nonspecific activation, and a higher bivalent affinity for p95HER2 of about 9 nM. When the antigen-specific receptor specifically binds p95HER2, the disclosure provides for a modified immune cell that is further modified so that it secretes a soluble (non-membrane-bound) bispecific antibody, e.g. BiTE (bispecific soluble antibody), that binds to both HER2 and a T cell activation antigen, e.g. CD3 epsilon or the constant chain (alpha or beta) of a TCR. Expressing the bispecific antibody may treat heterogeneous tumors that express both p95HER and HER2, and/or may mitigate effects of potential tumor cell escape through p95HER2 antigen loss following treatment with CAR-T cells targeting p95HER2. See, e.g., Choi et al., “CAR-T cells secreting BiTEs circumvent antigen escape without detectable toxicity,” *Nature Biotechnology*, 37:1049–1058 (2019). Other antigens include orphan tyrosine kinase receptor ROR1, tEGFR, Her2, p95HER2, LI-CAM, CD19, CD20, CD22, mesothelin, CEA, Claudin 18.2, hepatitis B surface antigen, anti-folate receptor, CD23, CD24, CD30, CD33, CD38, CD44, CD70, EGFR, EGP-2, EGP-4, EPHA2, ErbB2, 3, or 4, FBP, FcRH5 fetal acetylcholine e receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-13R-alpha2, kdr, kappa light chain, Lewis Y, Ll-cell adhesion molecule, MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, ROR1, TAG72, VEGF-R2, carcinoembryonic antigen (CEA), prostate specific antigen, PSMA, Her2/neu, p95HER2, estrogen receptor, progesterone receptor, ephrinB2, CD 123, CS-1

, c-Met, GD-2, and MAGE A3, CE7, Wilms Tumor 1 (WT-1), a cyclin, such as cyclin A1 (CCNA1), and/or molecules expressed by HIV, HCV, HBV or other pathogens.

[00140] Beside antigens from nonmutated canonical proteins overexpressed in cancer patients, cancer antigens according to the present invention also include neoantigens, generated by cancer-specific mutations as well as noncanonical polypeptides that can be generated without the need for somatic mutations.

[00141] Noncanonical cancer or tumor polypeptides include for example peptides derived from TE (transposable elements) such as in Bonté PE, Arribas YA, Merlotti A, et al. Single-cell RNA-seq-based proteogenomics identifies glioblastoma-specific transposable elements encoding HLA-I-presented peptides. *Cell Rep.* 2022;39(10):110916 ; LTR elements as in Attig J, Young GR, Hosie L, et al. LTR retroelement expansion of the human cancer transcriptome and immunopeptidome revealed by de novo transcript assembly. *Genome Res.* 2019;29(10):1578-1590 ; mid exon splicing such as in Kahles A, Lehmann KV, Toussaint NC, Hüser M, Stark SG, Sachsenberg T, Stegle O, Kohlbacher O, Sander C; Cancer Genome Atlas Research Network, et al. 2018. Comprehensive analysis of alternative splicing across tumors from 8,705 patients. *Cancer Cell* 34: 211–224.e6 ; intron retention such as in Smart AC, Margolis CA, Pimentel H, He MX, Miao D, Adeegbe D, Fugmann T, Wong KK, Van Allen EM. 2018. Intron retention is a source of neoepitopes in cancer. *Nat Biotechnol* 36: 1056–1058. 10.1038/nbt.4239, non-canonical splicing junctions such as described in Merlotti A, Sadacca B, Arribas YA, et al. Noncanonical splicing junctions between exons and transposable elements represent a source of immunogenic recurrent neo-antigens in patients with lung cancer. *Sci Immunol.* 2023;8(80):eabm6359 or in Shah NM, Jang HJ, Liang Y, et al. Pan-cancer analysis identifies tumor-specific antigens derived from transposable elements. *Nat Genet.* 2023;55(4):631-639). In some embodiments, the tumor antigen is selected from a intracellular peptide as described in WO 2022/189626 or WO 2022/189639. In these embodiments the antigen binding domain of the antigen receptor as herein described is typically a pMHC restricted antibody (e.g. a monoclonal antibody or mAbs) or a fragment thereof. In other embodiment, the tumor antigen is a surface antigen and notably a non-canonical surface polypeptide as defined for example in WO 2022/189620. More particularly, In some embodiments the extracellular antigen-binding domain binds to any of the tumor neoantigenic peptides disclosed in Int'l Pat. Pub. No. WO 2021/043804, incorporated by reference herein in its entirety. For example, the antigen-binding domain binds to any of the

peptides of SEQ ID NO: 1-117 or to a neoantigenic peptide comprising at least 8, 9, 10, 11 or 12 amino acids that is encoded by a part of an open reading frame (ORF) of any of the fusion transcript sequences of any one of SEQ ID NO: 118-17492 of WO 2021/043804 or described in any of WO 2018/234367, WO 2022/189620, WO 2022/189626, and WO-2022/189639.

5 [00142] In some embodiments the targeted antigen antigen has a low density on the cell surface, typically the antigen has a low density on the cell surface, typically of less than about 10,000 molecules per cell, e.g. less than about 5,000, 4,000, 3,000, 2,000, 1,000, 500, 250 or 100 molecules per cell. In some embodiments, the antigen is expressed at low density by the target cell, e.g., less than about 6,000 molecules of the target antigen per cell. In some embodiments, 10 the antigen is expressed at a density of less than about 5,000 molecules, less than about 4,000 molecules, less than about 3,000 molecules, less than about 2,000 molecules, less than about 1,000 molecules, or less than about 500 molecules of the target antigen per cell. In some embodiments, the antigen is expressed at a density of less than about 2,000 molecules, such as e.g., less than about 1,800 molecules, less than about 1,600 molecules, less than about 1,400 15 molecules, less than about 1,200 molecules, less than about 1,000 molecules, less than about 800 molecules, less than about 600 molecules, less than about 400 molecules, less than about 200 molecules, or less than about 100 molecules of the target antigen per cell. In some embodiments, the antigen is expressed at a density of less than about 1,000 molecules, such as e.g., less than about 900 molecules, less than about 800 molecules, less than about 700 molecules, less than 20 about 600 molecules, less than about 500 molecules, less than about 400 molecules, less than about 300 molecules, less than about 200 molecules, or less than about 100 molecules of the target antigen per cell. In some embodiments, the antigen is expressed at a density ranging from about 5,000 to about 100 molecules of the target antigen per cell, such as e.g., from about 5,000 to about 1,000 molecules, from about 4,000 to about 2,000 molecules, from about 3,000 to about 2,000 25 molecules, from about 4,000 to about 3,000 molecules, from about 3,000 to about 1,000 molecules, from about 2,000 to about 1,000 molecules, from about 1,000 to about 500 molecules, from about 500 to about 100 molecules of the target antigen per cell..

[00143] In any of the aspects or embodiments herein, the antigen-binding domain may bind the target antigen with a binding affinity K_d of 10^{-7} M or less, or 10^{-8} M or less, or 10^{-9} M or less 30 (smaller numbers indicating higher affinity).

Expression cassettes, vectors and targeting constructs

[00144] In some aspects, the genetic engineering involves introduction of a nucleic acid encoding the genetically engineered component or other component for introduction into the cell, such as a component encoding a gene-disruption protein or nucleic acid.

5 [00145] Generally, the engineering of CARs into immune cells (e.g., T cells) requires that the cells be cultured to allow for transduction and expansion. The transduction may utilize a variety of methods, but stable gene transfer is required to enable sustained CAR expression in clonally expanding and persisting engineered cells.

10 [00146] In some embodiments, gene transfer is accomplished by first stimulating cell growth, e.g., T cell growth, proliferation, and/or activation, followed by transduction of the activated cells, and expansion in culture to numbers sufficient for clinical applications.

[00147] Traditional techniques have utilized a suitable expression vector, in which case the immune cells are transduced with an expression cassette comprising a transgene, for example, an exogenous nucleic acid encoding a CAR or a modified TCR. Typically, the transgene or the expression cassette is cloned into a targeting construct, which provides for targeted integration of the expression cassette or the transgene at a targeted site within the genome (e.g., at a SUV39H1 locus).

20 [00148] Any suitable targeting construct suitable for expression in a cell of the invention, particularly an immune cell, can be employed. In particular embodiments, the targeting construct is compatible for use with a homologous recombination system suitable for targeted integration of the nucleic acid sequence (transgene) at a site (e.g., a SUV39H1 locus) within the genome of the cell.

[00149] Known vectors include viral vectors and pseudotyped viral vectors, such as retrovirus (e.g., moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rous sarcoma virus), lentivirus, adenovirus, adeno-associated virus (AAV), alphavirus, vaccinia virus, poxvirus, SV40-type viruses, polyoma viruses, Epstein-Barr viruses, herpes simplex virus, papilloma virus, polio virus, foamivirus, or Semliki Forest virus vectors; or transposase systems, such as Sleeping Beauty transposase vectors (see, e.g., Koste et al. (2014) Gene Therapy 2014 Apr 3.; Carlens et al. (2000) Exp Hematol 28(10): 1137-46; Alonso-Camino et al. (2013) Mol Ther Nucl Acids 2, e93; Park et al., Trends Biotechnol. 2011

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November; 29(11): 550-557). A number of illustrative retroviral systems have been described (e.g., U.S. Pat. Nos. 5,219,740; 6,207,453; 5,219,740; Miller and Rosman (1989) BioTechniques 7:980-990; Miller, A. D. (1990) Human Gene Therapy 1:5-14; Scarpa et al. (1991) Virology 180:849-852; Burns et al. (1993) Proc. Natl. Acad. Sci. USA 90:8033-8037; and Boris-Lawrie and Temin (1993) Cur. Opin. Genet. Develop. 3: 102-109.

[00150] Methods of lentiviral transduction are also known. Exemplary methods are described in, e.g., Wang et al. (2012) J. Immunother. 35(9): 689-701; Cooper et al. (2003) Blood. 101: 1637-1644; Verhoeven et al. (2009) Methods Mol Biol. 506: 97-114; and Cavalieri et al. (2003) Blood. 102(2): 497-505.

[00151] Non-viral systems for delivery of naked plasmids to cells include lipofection, nucleofection, microinjection, biolistics, virosomes, lipids, cationic lipid complexes, liposomes, immunoliposomes, nanoparticle, gold particle, or polymer complex, poly-lysine conjugates, synthetic polyamino polymers, other agent-enhanced uptake of DNA, and artificial viral envelopes or virions.

[00152] In some embodiments, recombinant nucleic acids are transferred into T cells via electroporation {see, e.g., Chicaybam et al, (2013) PLoS ONE 8(3): e60298 and Van Tedeloo et al. (2000) Gene Therapy 7(16): 1431-1437). In some embodiments, recombinant nucleic acids are transferred into T cells via transposition (see, e.g., Manuri et al. (2010) Hum Gene Ther 21(4): 427-437; Sharma et al. (2013) Molec Ther Nucl Acids 2, e74; and Huang et al. (2009) Methods Mol Biol 506: 115-126). Other methods of introducing and expressing genetic material in immune cells include calcium phosphate transfection (e.g., as described in Current Protocols in Molecular Biology, John Wiley & Sons, New York. N.Y.), protoplast fusion, cationic liposome-mediated transfection; tungsten particle-facilitated microparticle bombardment (Johnston, Nature, 346: 776-777 (1990)); and strontium phosphate DNA co-precipitation (Brash et al., Mol. Cell Biol., 7: 2031-2034 (1987)).

[00153] Particularly useful vectors for generating a target construct that provides transgene vectorization for homologous recombination-mediated targeting include, but are not limited to, recombinant Adeno- Associated Virus (rAAV), recombinant non-integrating lentivirus (rNILV), recombinant non-integrating gamma-retrovirus (rNIgRV), single-stranded DNA (linear or circular), and the like. Such vectors can be used to introduce a transgene into an immune cell of

the invention by making a targeting construct (see, for example, Miller, Hum. Gene Ther. 1(1):5-14 (1990); Friedman, Science 244: 1275-1281 (1989); Eglitis et al., BioTechniques 6:608-614 (1988); Tolstoshev et al., Current Opin. Biotechnol. 1 :55-61 (1990); Sharp, Lancet 337: 1277-1278 (1991); Cornetta et al., Prog. Nucleic Acid Res. Mol. Biol. 36:311-322 (1989); Anderson, Science 226:401-409 (1984); Moen, Blood Cells 17:407-416 (1991); Miller et al., Biotechnology 7:980-990 (1989); Le Gal La Salle et al., Science 259:988-990 (1993); and Johnson, Chest 107:77S- 83S (1995); Rosenberg et al., N. Engl. J. Med. 323 :370 (1990); Anderson et al., U.S. Pat. No. 5,399,346; Scholler et al., Sci. Transl. Med. 4: 132-153 (2012; Parente-Pereira et al., J. Biol. Methods 1(2):e7 (1-9)(2014); Lamers et al., Blood 117(1):72-82 (2011); Reviere et al., Proc. Natl. Acad. Sci. USA 92:6733-6737 (1995); Wang et al., Gene Therapy 15: 1454-1459 (2008)).

[00154] In some embodiments, the exogenous nucleic acid or the targeting construct comprises a 5' homology arm and a 3' homology arm to promote recombination of the nucleic acid sequence into the cell genome at the nuclease cleavage site.

[00155] In some embodiments, an exogenous nucleic acid can be introduced into the cell using a single-stranded DNA template. The single-stranded DNA can comprise the exogenous nucleic acid and, in preferred embodiments, can comprise 5' and 3' homology arms to promote insertion of the nucleic acid sequence into the nuclease cleavage site by homologous recombination. The single-stranded DNA can further comprise a 5' AAV inverted terminal repeat (ITR) sequence 5' upstream of the 5' homology arm, and a 3' AAV ITR sequence 3' downstream of the 3' homology arm. In other particular embodiments, the targeting construct comprises in 5' to 3' order: a first viral sequence, a left homology arm, a nucleic acid sequence encoding a self-cleaving porcine teschovirus 2A, a transgene, a polyadenylation sequence, a right homology arm and a second viral sequence. In a preferred embodiment, the targeting construct comprises in 5' to 3' order: a first viral sequence, a left homology arm, a nucleic acid sequence encoding a self-cleaving porcine teschovirus 2A, a nucleic acid sequence encoding a CAR, a polyadenylation sequence, a right homology arm and a second viral sequence. Another suitable targeting construct can comprise sequences from an integrative-deficient Lentivirus (see, for example, Wanisch et al., Mol. Ther. 17(8): 1316-1332 (2009)). In a particular embodiment, the viral nucleic acid sequence comprises sequences of an integrative-deficient Lentivirus. It is understood that any suitable targeting construction compatible with a homologous recombination system employed can be utilized. The AAV nucleic acid sequences that function as part of a targeting construct can be packaged in

several natural or recombinant AAV capsids or particles. In a particular embodiment, the AAV particle is AAV6. In a particular embodiment, an AAV2 -based targeting construct is delivered to the target cell using AAV6 viral particles. In a particular embodiment, the AAV sequences are AAV2, AAV5 or AAV6 sequences.

5 [00156] In some embodiments, the gene encoding an exogenous nucleic acid sequence of the invention can be introduced into the cell by transfection with a linearized DNA template. In some examples, a plasmid DNA encoding an exogenous nucleic acid sequence can include nuclease cleavage site (such as class II, type II, V or VI Cas nuclease) at both sides of the left homology arm such that the circular plasmid DNA is linearized and allows precise in-frame integration of
10 exogenous DNA without backbone vector sequences (see for example Hisano Y, Sakuma T, Nakade S, et al. Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. *Sci Rep.* 2015;5:8841).

[00157] Well-suited constructs including a modified TCR typically include a TRBC or TRAC sequence (which can be a native or modified TRBC or TRAC sequence, including murine
15 sequences as described herein), a cleavable linker sequence (as defined above, but such as a 2A sequence), a TRAC and/or TRBC sequence (which can be a native or modified TRBC and/or TRAC sequence, including murine sequences as described herein). The TRBC and/or the TRAC sequence is typically fused (preferably in 5') to a sequence coding for an antibody fragment as above described (e.g. a VH, a VH, an scFv, a single domain antibody, a VHH, etc a previously
20 exemplified). Typically, the booster (co-stimulatory ligand) sequence is included in the same construct, such that in preferred embodiments, the construct further includes a cleavable linker sequence (e.g. a 2A sequence) and a booster (co-stimulatory ligand) sequence. In some embodiments, the TRAC and/or TRBC sequence in the 3' end of the construct is fused to a cleavable linker which is also fused to the booster (co-stimulatory ligand and or costimulatory
25 receptor CCR) sequence. The booster ((co-stimulatory ligand and/or costimulatory receptor CCR) sequence can be any one as herein described and can be notably a CD80 sequence or a CD80_4-1BBL sequence as herein described.

[00158] In some embodiments, the vector incorporates an endogenous promoter such as a TCR promoter. Such a vector could provide for expression in a manner similar to that provided by an
30 endogenous promoter, such as a TCR promoter. Such a vector can be useful, for example, if the

site of integration does not provide for efficient expression of a transgene, or if disruption of the endogenous gene controlled by the endogenous promoter would be detrimental to the T cell or would result in a decrease in its effectiveness in T cell therapy. In a preferred embodiment, such a vector can be useful, for example, if the site of integration does not provide for efficient expression of nucleic acid sequence encoding a CAR or a modified TCR. The promoter can be an inducible promoter or a constitutive promoter. Expression of a nucleic acid sequence under the control of an endogenous or vector-associated promoter occurs under suitable conditions for the cell to express the nucleic acid, for example, growth conditions, or in the presence of an inducer with an inducible promoter, and the like. Such conditions are well understood by those skilled in the art.

[00159] The targeting construct can optionally be designed to include a P2A sequence directly upstream of the nucleic acid sequences encoding the transgene. In a preferred embodiment, the targeting construct can optionally be designed to include a P2A sequence directly upstream of the nucleic acid sequences encoding a therapeutic protein (e.g. an engineered antigen receptor). P2A is a self-cleaving peptide sequence, which can be used for bicistronic or multicistronic expression of protein sequences (see Szymczak et al., Expert Opin. Biol. Therapy 5(5):627-638 (2005)). If desired, the targeting construct can optionally be designed to include a reporter, for example, a reporter protein that provides for identification of transduced cells. Exemplary reporter proteins include, but are not limited to, fluorescent proteins, such as mCherry, green fluorescent protein (GFP), blue fluorescent protein, for example, EBFP, EBFP2, Azurite, and mKalamal, cyan fluorescent protein, for example, ECFP, Cerulean, and CyPet, and yellow fluorescent protein, for example, YFP, Citrine, Venus, and YPet. Typically, the targeting construct comprises a polyadenylation (poly A) sequence 3' of the transgene. In a preferred embodiment, the targeting construct comprises a polyadenylation (poly A) sequence 3' of the nucleic acid sequences encoding a CAR.

Methods for genetically engineering an immune cell of the invention

[00160] The present invention provides a method for producing a genetically-modified immune cell comprising an exogenous nucleic acid of interest inserted at a Suv39H1 locus of the said immune cell. The method comprises introducing into the said immune cell one or more nucleic acids including: (a) a nuclease or a first nucleic acid sequence encoding a nuclease

described herein, wherein the nuclease is expressed in the immune cell; and (b) a second nucleic acid sequence including a nucleic acid (therapeutic transgene) coding for at least one therapeutic protein; wherein the nuclease produces a cleavage site in the SUV39H1 locus at a recognition sequence (which can be an intron or an exon) of the SUV39H1 gene; and wherein the exogenous nucleic acid of interest (i.e. the therapeutic transgene) is inserted at the cleavage site. In some embodiments, typically when the sequence of interest is inserted in an exon, the transgene is inserted in frame. Alternatively, notably when the transgene is inserted in an intron (typically upstream exon 1) the transgene may include an exogenous splice acceptor site and/or a poly A signal.

10 *Gene editing agents for site specific cleavage*

[00161] Inactivation of SUV39H1 in a cell according to the invention may be effected via repression or disruption of the SUV39H1 gene, such as by deletion, e.g., deletion of the entire gene, exon, or region, and/or replacement with an exogenous sequence, and/or frameshift within the gene, typically within an exon of the gene. In some embodiments, the disruption results in a premature stop codon being incorporated into the gene, such that the SUV39H1 protein is not expressed or is non-functional. The disruption is generally carried out at the DNA level. The disruption generally is permanent, irreversible, or not transient.

[00162] In some embodiments, the gene inactivation is achieved using gene editing agents such as a DNA-targeting molecule, such as a DNA-binding protein or DNA-binding nucleic acid, or complex, compound, or composition, containing the same, which specifically binds to or hybridizes to the gene that allows site specific cleavage. In some embodiments, the DNA-targeting molecule comprises a DNA-binding domain, e.g., a zinc finger protein (ZFP) DNA-binding domain, a transcription activator-like protein (TAL) or TAL effector (TALE) DNA-binding domain, a clustered regularly interspaced short palindromic repeats (CRISPR) DNA-binding domain, or a DNA-binding domain from a meganuclease.

[00163] Zinc finger, TALE, and CRISPR system binding domains can be "engineered" to bind to a predetermined nucleotide sequence.

[00164] In some embodiments, the DNA-targeting molecule, complex, or combination contains a DNA-binding molecule and one or more additional domain, such as an effector domain to facilitate the repression or disruption of the gene. For example, in some embodiments, the gene

disruption is carried out by fusion proteins that comprise DNA-binding proteins and a heterologous regulatory domain or functional fragment thereof.

[00165] Typically, the additional domain is a nuclease domain. Thus, in some embodiments, gene disruption is facilitated by gene or genome editing, using engineered proteins, such as nucleases and nuclease-containing complexes or fusion proteins, composed of sequence-specific DNA-binding domains fused to, or complexed with, non-specific DNA-cleavage molecules such as nucleases.

[00166] These targeted chimeric nucleases or nuclease-containing complexes carry out precise genetic modifications by inducing targeted double-stranded breaks or single-stranded breaks, stimulating the cellular DNA-repair mechanisms, including error-prone nonhomologous end joining (NHEJ) and homology-directed repair (HDR). In some embodiments the nuclease is an endonuclease, such as a zinc finger nuclease (ZFN), TALE nuclease (TALEN), an RNA-guided endonuclease (RGEN), such as a CRISPR-associated (Cas) protein, or a meganuclease. Such systems are well-known in the art (see, for example, U.S. Pat. No. 8,697,359; Sander and Joung (2014) Nat. Biotech. 32:347-355; Hale et al. (2009) Cell 139:945-956; Karginov and Hannon (2010) Mol. Cell 37:7; U.S. Pat. Publ. 2014/0087426 and 2012/0178169; Boch et al. (2011) Nat. Biotech. 29: 135-136; Boch et al. (2009) Science 326: 1509-1512; Moscou and Bogdanove (2009) Science 326: 1501; Weber et al. (2011) PLoS One 6:e19722; Li et al. (2011) Nucl. Acids Res. 39:6315-6325; Zhang et al. (2011) Nat. Biotech. 29: 149-153; Miller et al. (2011) Nat. Biotech. 29: 143-148; Lin et al. (2014) Nucl. Acids Res. 42:e47). Such genetic strategies can use constitutive expression systems or inducible expression systems according to well-known methods in the art.

ZFPs and ZFNs; TALs, TALEs, and TALENs

[00167] In some embodiments, the DNA-targeting molecule includes a DNA-binding protein such as one or more zinc finger protein (ZFP) or transcription activator-like protein (TAL), fused to an effector protein such as an endonuclease. Examples include ZFNs, TALEs, and TALENs. See Lloyd et al., Frontiers in Immunology, 4(221), 1-7 (2013).

[00168] In some embodiments, the DNA-targeting molecule comprises one or more zinc-finger proteins (ZFPs) or domains thereof that bind to DNA in a sequence-specific manner. A ZFP or domain thereof is a protein or domain within a larger protein, that binds DNA in a

sequence-specific manner through one or more zinc fingers regions of amino acid sequence within the binding domain whose structure is stabilized through coordination of a zinc ion. Generally, sequence-specificity of a ZFP may be altered by making amino acid substitutions at the four helix positions (-1, 2, 3 and 6) on a zinc finger recognition helix. Thus, in some embodiments, the ZFP or ZFP-containing molecule is non-naturally occurring, e.g., is engineered to bind to the target site of choice. See, for example, Beerli et al. (2002) *Nature Biotechnol.* 20: 135-141; Pabo et al. (2001) *Ann. Rev. Biochem.* 70:313-340; Isalan et al. (2001) *Nature Biotechnol.* 19:656-660; Segal et al. (2001) *Curr. Opin. Biotechnol.* 12:632-637; Choo et al. (2000) *Curr. Opin. Struct. Biol.* 10:411-416.

[00169] In some embodiments, the DNA-targeting molecule is or comprises a zinc-finger DNA binding domain fused to a DNA cleavage domain to form a zinc-finger nuclease (ZFN). In some embodiments, fusion proteins comprise the cleavage domain (or cleavage half-domain) from at least one Type IIS restriction enzyme and one or more zinc finger binding domains, which may or may not be engineered. In some embodiments, the cleavage domain is from the Type IIS restriction endonuclease Fok I. See, for example, U.S. Pat. Nos. 5,356,802; 5,436,150 and 5,487,994; as well as Li et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:4275-4279; Li et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:2764-2768; Kim et al. (1994a) *Proc. Natl. Acad. Sci. USA* 91:883-887; Kim et al. (1994b) *J. Biol. Chem.* 269:31,978-31,982.

[00170] In some aspects, the ZFNs efficiently generate a double strand break (DSB), for example at a predetermined site in the coding region of the targeted gene (i.e. SUV39H1). Typical targeted gene regions include exons, regions encoding N-terminal regions, first exon, second exon, and promoter or enhancer regions. In some embodiments, transient expression of the ZFNs promotes highly efficient and permanent disruption of the target gene in the engineered cells. In particular, in some embodiments, delivery of the ZFNs results in the permanent disruption of the gene with efficiencies surpassing 50%. Many gene-specific engineered zinc fingers are available commercially. For example, Sangamo Biosciences (Richmond, CA, USA) has developed a platform (CompoZr) for zinc-finger construction in partnership with Sigma-Aldrich (St. Louis, MO, USA), allowing investigators to bypass zinc-finger construction and validation altogether, and provides specifically targeted zinc fingers for thousands of proteins. Gaj et al., *Trends in Biotechnology*, 2013, 31(7), 397-405. In some embodiments, commercially available zinc fingers

are used or are custom designed. (See, for example, Sigma-Aldrich catalog numbers CSTZFND, CSTZFN, CTI1-1KT, and PZD0020).

[00171] In some embodiments, the DNA-targeting molecule comprises a naturally occurring or engineered (non-naturally occurring) transcription activator-like protein (TAL) DNA binding domain, such as in a transcription activator-like protein effector (TALE) protein, See, e.g., U.S. Patent Publication No. 20110301073. In some embodiments, the molecule is a DNA binding endonuclease, such as a TALE-nuclease (TALEN). In some aspects the TALEN is a fusion protein comprising a DNA-binding domain derived from a TALE and a nuclease catalytic domain to cleave a nucleic acid target sequence. In some embodiments, the TALE DNA-binding domain has been engineered to bind a target sequence within genes that encode the target antigen and/or the immunosuppressive molecule. For example, in some aspects, the TALE DNA-binding domain may target CD38 and/or an adenosine receptor, such as A2AR.

[00172] In some embodiments, the TALEN recognizes and cleaves the target sequence in the gene. In some aspects, cleavage of the DNA results in double-stranded breaks. In some aspects the breaks stimulate the rate of homologous recombination or non-homologous end joining (NHEJ). Generally, NHEJ is an imperfect repair process that often results in changes to the DNA sequence at the site of the cleavage. In some aspects, repair mechanisms involve rejoining of what remains of the two DNA ends through direct re-ligation (Critchlow and Jackson, Trends Biochem Sci. 1998 Oct;23(10):394-8) or via the so-called microhomology-mediated end joining. In some embodiments, repair via NHEJ results in small insertions or deletions and can be used to disrupt and thereby repress the gene. In some embodiments, the modification may be a substitution, deletion, or addition of at least one nucleotide. In some aspects, cells in which a cleavage-induced mutagenesis event, i.e. a mutagenesis event consecutive to an NHEJ event, has occurred can be identified and/or selected by well-known methods in the art.

[00173] TALE repeats can be assembled to specifically target the SUV39H1 gene. (Gaj et al., Trends in Biotechnology, 2013, 31(7), 397-405). A library of TALENs targeting 18,740 human protein-coding genes has been constructed (Kim et al., Nature Biotechnology. 31, 251-258 (2013)). Custom-designed TALE arrays are commercially available through Collectis Bioresearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA), and Life Technologies (Grand Island, NY, USA). Specifically, TALENs that target CD38 are

commercially available (See Gencopoeia, catalog numbers HTN222870-1, HTN222870-2, and HTN222870-3, available on the World Wide Web at www.genecopoeia.com/product/search/detail.php?prt=26&cid=&key=HTN222870). Exemplary molecules are described, e.g., in U.S. Patent Publication Nos. US 2014/0120622, and 2013/0315884.

[00174] In some embodiments the TALENs are introduced as transgenes encoded by one or more plasmid vectors. In some aspects, the plasmid vector can contain a selection marker which provides for identification and/or selection of cells which received said vector.

RGENs (CRISPR/Cas systems)

[00175] The gene repression can be carried out using one or more DNA -binding nucleic acids, such as disruption via an RNA-guided endonuclease (RGEN), or other form of repression by another RNA-guided effector molecule. For example, in some embodiments, the gene repression can be carried out using clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins. See Sander and Joung, *Nature Biotechnology*, 32(4): 347-355.

[00176] In general, "CRISPR system" refers collectively to transcripts and other elements involved in the expression of, or directing the activity of, CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), and/or other sequences and transcripts from a CRISPR locus.

[00177] Typically, the CRISPR/Cas nuclease or CRISPR/Cas nuclease system includes a non-coding RNA molecule (guide) RNA, which sequence-specifically binds to DNA, and a CRISPR protein, with nuclease functionality (e.g., two nuclease domains).

[00178] In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of the target sequence. Typically, in the context of formation of a CRISPR complex, "target sequence" generally refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between the target sequence and a guide sequence promotes the formation of a CRISPR complex. Full complementarity is not necessarily required, provided there is sufficient complementarity to cause hybridization and

promote formation of a CRISPR complex. The target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. Generally, a sequence or template that may be used for recombination into the targeted locus comprising the target sequences is referred to as an "editing template" or "editing polynucleotide" or "editing sequence". In some aspects, an exogenous
5 template polynucleotide may be referred to as an editing template. In some aspects, the recombination is homologous recombination.

[00179] In some embodiments, one or more vectors driving expression of one or more elements of the CRISPR system are introduced into the cell such that expression of the elements of the CRISPR system direct formation of the CRISPR complex at one or more target sites. For
10 example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. In some embodiments, CRISPR system
15 elements that are combined in a single vector may be arranged in any suitable orientation. In some embodiments, the CRISPR enzyme, guide sequence, tracr-mate sequence, and tracr sequence are operably linked to and expressed from the same promoter. In some embodiments, a vector comprises a regulatory element operably linked to an enzyme-coding sequence encoding the CRISPR enzyme, such as a Cas protein.

[00180] In some embodiments, the Cas protein has nuclease activity. For example, the Cas protein can modify the target nucleic acid by cleaving the target nucleic acid. The cleaved target nucleic acid can then undergo homologous recombination with a nearby HDRT. For example, the Cas protein can direct cleavage of one or both strands at a location in a target nucleic acid. Non-limiting examples of Cas proteins include CasI, CasIB, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8,
25 Cas9 (also known as CsnI and CsxI2), CasIO, CsyI, Csy2, Csy3, CseI, Cse2, CseI, Cse2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, CmrI, Cmr3, Cmr4, Cmr5, Cmr6, CsbI, Csb2, Csb3, CsxI7, CsxI4, CsxIO, CsxI6, CsaX, Csx3, CsxI, CsxI5, CsfI, Csf2, Csf3, Csf4, CpfI, homologs thereof, variants thereof, mutants thereof, and derivatives thereof. There are three main types of Cas proteins (type I, type II, and type III), and 10 subtypes including 5 type I, 3 type II, and 2
30 type III proteins (see, e.g., Hochstrasser and Doudna, Trends Biochem Sci, 2015:40(1):58-66).

Type II Cas proteins include Cas1, Cas2, Csn2, Cas9, and Cpf1. These Cas proteins are known to those skilled in the art.

[00181] The CRISPR/Cas9 technology originates from type II CRISPR/Cas systems, which consist of one DNA endonuclease protein, Cas9, and two small RNAs, CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The small RNAs or a chimeric single guide RNA (sgRNA) bind Cas9, thus forming an RNA-guided DNA endonuclease (RGEN) complex, and cleave a specific. For example, the amino acid sequence of the *Streptococcus pyogenes* wild-type Cas9 polypeptide is set forth, e.g., in NCBI Ref. Seq. No. NP_269215, and the amino acid sequence of *Streptococcus thermophilus* wild-type Cas9 polypeptide is set forth, e.g., in NCBI Ref. Seq. No. WP_011681470.

[00182] In some embodiments Cas9 CRISPR guide that hybridizes with Suv39h1 genomic nucleic acid sequence having a sequence selected from the table below can be used according to the present invention.

gRNA name	SUV39H1 Exon	Targeting sequence	PAM	Position	Strand
gRNA-SUV-1	2	GGTTCCTCTTAGAGATACCG (SEQ ID NO: 3)	AGG	48698980	-
gRNA-SUV-2	3	GATATCCACGCCATTTACCC (SEQ ID NO: 4)	AGG	48700107	-
gRNA-SUV-3	3	TTGATGTACACGAAGGCCCG (SEQ ID NO: 5)	CGG	48700396	-
gRNA-SUV-4	3	GCATCTTCCGCACGGATGAT (SEQ ID NO: 6)	GGG	48700661	+
gRNA-SUV-5	3	TCGCAAGAACAGCTTCGTCA (SEQ ID NO: 7)	TGG	48700716	+

[00183] In certain embodiments, the Cas nuclease is a type V-A, type V-C, or type V-D Cas nuclease. In certain embodiments, the Cas nuclease is a type V-A nuclease such as Cpf1 is another type of RGEN derived from the type V CRISPR system. The Cpf1 enzyme can be derived from any genera of microbes including but not limited to *Parvobacteria*, *Lachnospiraceae*, *Butyrivibrio*, *Peregrinibacteria*, *Acidaminococcus*, *Porphyromonas*, *Lachnospiraceae*, *Porphyromonas*,

Prevotella, Moraxella, Smithella, Leptospira, Lachnospiraceae, Francisella, Candidatus, and Eubacterium. In one embodiment, Cpf1 is derived from a species from the Acidaminococcus genus (AsCpf1). In another embodiment, Cpf1 is derived from a species from the Lachnospiraceae genus (LbCpf1). Cpf1 Cas proteins and variants thereof have been described for example in Zetsche B, Gootenberg JS, Abudayyeh OO, et al. *Cell*. 2015;163(3):759-771 but relevant cpf1 Cas proteins are for example described in U.S. Patent No 9,790,490, WO-2021118626, WO-2021119563, WO-2018236548, WO-2018191715, WO-2017189308, EP3835418, US20210254038, US20180148735, US20170233756 or US20190071688.

Donor template

[00184] The invention provides for the simultaneous introduction of an exogenous nucleic acid into the cell, such that the exogenous nucleic acid sequence or transgene is inserted into the SUV39H1 region gene at the nuclease cleavage site. The transgene typically includes a nucleic acid sequence encoding a therapeutic protein as defined herein. Typically, in the present invention, a transgene is cloned into a targeting construct, which provides for targeted integration of the transgene at a site within the genome. Any suitable targeting construct suitable for expression in a cell of the invention, particularly a human T cell, can be employed. Transgene insertion at the nuclease cleavage site can be achieved in various dependent methods that are well summarized in Lau CH, Tin C, Suh Y. CRISPR-based strategies for targeted transgene knock-in and gene correction. *Fac Rev*. 2020;9:20. In particular, various CRISPR-mediated homology-dependent and -independent gene knock-in and gene correction strategies have been developed including Cas nucleases variants such as Cas9 variants (for example, Cas9 nuclease, Cas9 nickase, Cpf1 nuclease, CasX, dCas9, or dCas13), fusion domains (for example, HDR enhancer, base editor, splicing effector, polymerase, or RT), template donor type (for example, plasmid DNA, ssODN, PCR fragments, or homologous chromosome), length of homology arms (for example, none, 5–80 bp, or 500–1000 bp), number of guide RNAs (for example, single-cut or double-cut on donor and genomic DNA), DNA cleavage (for example, double-strand break, single-strand nick, or no cleavage), and cleavage pattern (for example, blunt or staggered cut).

[00185] In some embodiments, the targeting construct is compatible for use with a homologous recombination system suitable for targeted integration of the nucleic acid sequence (transgene) at a site within the genome of the cell. HDR mediated by homologous recombination is one of the

most commonly used methods to introduce a genetic mutation into the genome (gene knock-in). It allows desired and controlled genetic modifications in the genome. HDR requires a repair template, either endogenous or exogenous, to transfer the sequence information from the repair template (i.e. the targeting construct) to the target gene. As used herein, the term “donor template” refers to a nucleic acid designed to serve as a repair template at or near the target nucleotide sequence upon introduction into a cell or organism. In certain embodiments, the donor template is complementary to a polynucleotide comprising the target nucleotide sequence (e.g., the SUV39H1 gene) or a portion thereof. When optimally aligned, a donor template may overlap with one or more nucleotides of a target nucleotide sequences (e.g. about or more than about 1, 5, 10, 15, 20, 25, 30, 35, 40, or more nucleotides). The nucleotide sequence of the donor template is typically not identical to the genomic sequence that it replaces as long as sufficient homology is present to support homology-directed repair. In certain embodiments, the donor template comprises a non-homologous sequence (i.e. a nucleic acid comprising a sequence encoding a therapeutic protein) flanked by two regions of homology (i.e., homology arms), such that homology-directed repair between the target DNA region and the two flanking sequences results in insertion of the non-homologous sequence at the target region.

[00186] In certain embodiments, the donor template comprises a non-homologous sequence 10-100 nucleotides, 50-500 nucleotides, 100-1,000 nucleotides, 200-2,000 nucleotides, or 500-5,000 nucleotides in length positioned between two homology arms.

[00187] In certain embodiments, where HDR of the non-target strand is desired, the donor template comprises a first homology arm homologous to a sequence 5' to the target nucleotide sequence and a second homology arm homologous to a sequence 3' to the target nucleotide sequence. In certain embodiments, the first homology arm is at least 50% (e.g. at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) identical to a sequence 5' to the target nucleotide sequence. In certain embodiments, the second homology arm is at least 50% (e.g. at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) identical to a sequence 3' to the target nucleotide sequence. In certain embodiments, when the donor template sequence and a polynucleotide comprising a target nucleotide sequence are optimally aligned, the nearest

nucleotide of the donor template is within about 1, 5, 10, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 1000, 2000, 3000, 4000, or more nucleotides from the target nucleotide sequence.

[00188] In certain embodiments, the donor template further comprises an engineered sequence not homologous to the sequence to be repaired. Such engineered sequence can harbor a barcode and/or a sequence capable of hybridizing with a donor template-recruiting sequence disclosed herein.

[00189] The donor template can be provided to the cell as single-stranded DNA, single-stranded RNA, double-stranded DNA, linear double-stranded DNA PCR fragments, ssODN, or double-stranded RNA. Typically, suitable CRISPR-Cas systems or other editing agents may possess nuclease activity to cleave the target strand, the non-target strand, or both. When HDR of the target strand is desired, a donor template having a nucleic acid sequence complementary to the target strand is also contemplated.

[00190] The donor template can be introduced into a cell in linear or circular form.

[00191] If introduced in linear form, the ends of the donor template may be protected (e.g., from exonucleolytic degradation) by methods known to those of skill in the art. For example, one or more dideoxynucleotide residues are added to the 3' terminus of a linear molecule and/or self-complementary oligonucleotides are ligated to one or both ends (see, for example, Chang et al. (1987) PROC. NATL. ACAD SCI USA, 84: 4959; Nehls et al (1996) SCIENCE, 272: 886; see also the chemical modifications for increasing stability and/or specificity of RNA disclosed supra). Additional methods for protecting exogenous polynucleotides from degradation include, but are not limited to, addition of terminal amino group(s) and the use of modified internucleotide linkages such as, for example, phosphorothioates, phosphoramidates, and O-methyl ribose or deoxyribose residues. As an alternative to protecting the termini of a linear donor template, additional lengths of sequence may be included outside of the regions of homology that can be degraded without impacting recombination.

[00192] To replace large genomic regions (for example, 58 kb) or integrate a very large transgene (for example, 200 kb) into the genome, two additional short ssODNs (~80 bp) can be introduced together with self-cleaving plasmid donor and CRISPR/Cas9 targeting the genomic locus into the cell. Upon double-strand breaks, these two ssODNs ligate each cut end to join the

genomic DNA and the plasmid donor via the SDSA repair pathway. This integrated approach eliminates the need of attaching homology arms to the donor vector.

[00193] It has been reported recently (Román-Rodríguez FJ, Ugalde L, Álvarez L, et al. *Cell Stem Cell*. 2019; 25(5): 607–621.e7; He X, Tan C, Wang F, et al. *Nucleic Acids Res*. 2016; 44(9): e85; Artegiani B, Hendriks D, Beumer J, et al. *Nat Cell Biol*. 2020; 22(3): 321–31) that NHEJ-based knock-in may have a higher efficiency than HDR-mediated gene targeting. In this case, double-strand DNA breaks were introduced to both the genome and donor template for mediating transgene insertion via the NHEJ repair pathway. The donor plasmid can be linearized using a Cas nuclease such as Cas9 to cleave one sgRNA target site (for the single-cut donor) or two sgRNA target sites at both sides of the transgene (for the double-cut donor). However, double-cut donor is less efficient than the single-cut donor because the former will generate two DNA fragments that compete for genomic integration. The linearized donor plasmid then is directly ligated to the broken genomic DNA ends upon NHEJ repair. NHEJ efficiently re-ligates DNA ends without mistakes and it does not require regions of homology for precise transgene insertion. To avoid misorientation and off target DNA double-strand breaks, a short homology DNA sequence bearing the Cas9 target sequence (bait sequence) can be introduced onto a donor plasmid (Auer TO, Duroure K, de Cian A, et al. *Genome Res*. 2014; 24(1): 142–53). In this case, concurrent cleavage of the target genomic locus and bait plasmid sequence leads to efficient targeted integration of a large transgene via NHEJ pathway. The insertion is independent from the homology sequence between the target locus and the bait in the donor plasmid. Similar NHEJ strategy can also be achieved by using Cpf1 to create sticky ends at the DNA cleavage site (see Lau CH, Tin C, Suh Y. CRISPR-based strategies for targeted transgene knock-in and gene correction. *Fac Rev*. 2020;9:20 for more details).

[00194] A donor template can be a component of a vector as described herein, contained in a separate vector, or provided as a separate polynucleotide, such as an oligonucleotide, linear polynucleotide, or synthetic polynucleotide. In certain embodiments, the donor template is a DNA. In certain embodiments, a donor template is in the same nucleic acid as a sequence encoding guide nucleic acid, and/or a sequence encoding the Cas protein, where applicable. In certain embodiments, a donor template is provided in a separate nucleic acid. A donor template polynucleotide may be of any suitable length, such as about or at least about 50, 75, 100, 150, 200, 500, 1000, 2000, 3000, 4000, or more nucleotides in length.

[00195] In some embodiments, the donor template is introduced into a cell as part of a vector (e.g., a plasmid) and can include additional sequences such as, for example, replication origins, promoters and genes encoding antibiotic resistance, that are not intended for insertion into the DNA region of interest. A donor template can be also be delivered by viruses (e.g., adenovirus, adeno-associated virus (AAV)). In certain embodiments, the donor template is introduced as an AAV, e.g. a pseudotyped AAV. The capsid proteins of the AAV can be selected by a person skilled in the art based upon the tropism of the AAV and the target cell type. For example, in certain embodiments, the donor template is introduced into a hematopoietic stem cell, a hematopoietic progenitor cell, or a T lymphocyte (e.g, CD8+ T lymphocyte) as AAV6 or an AAVHSC (see, U.S. Patent No, 9,890,396). In certain embodiments, a non-viral donor template is introduced into the target cell as a naked nucleic acid or in complex with a liposome or poloxamer. In certain embodiments, a non-viral donor template is introduced into the target cell by electroporation. In other embodiments, a viral donor template is introduced into the target cell by infection.

[00196] The engineered, non-naturally occurring system can be delivered before, after, or simultaneously with the donor template (see, International (PCT) Application Publication No. WO2017/053729).

[00197] In certain embodiments, the donor template is conjugated covalently to guide nucleic acid. Covalent linkages suitable for this conjugation are known in the art and are described, for example, in U.S. Patent No. 9,982,278 and Savic et al (2018) ELJFE 7:e33761. In certain embodiments, the donor template is covalently linked to the guide nucleic acid (e.g, the 5' end of the modulator nucleic acid) through an internucleotide bond. In certain embodiments, the donor template is covalently linked to the modulator nucleic acid (e.g., the 5' end of the modulator nucleic acid) through a linker.

Delivery of nucleic acids or polynucleotides, including vectors

[00198] Art-recognized techniques for introducing foreign nucleic acids (e.g., DNA and RNA) into a host cell, include calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, DNA-loaded liposomes, lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, biolistics, and viral-mediated transfection. Compositions comprising the

nucleic acids may also comprise transfection facilitating agents, which include surface active agents, quinone analogs, vesicles such as squalene and squalene, hyaluronic acid, lipids, liposomes, lecithin liposomes, calcium ions, viral proteins, polyanions, polycations, including poly-L-glutamate, or nanoparticles, gold particles, or other known agents. Delivery vehicles
5 include a liposome, lipid-containing complex, nanoparticle, gold particle, or polymer complex.

[00199] Lipid materials have been used to create lipid nanoparticles (LNPs) based on ionizable cationic lipids, which exhibit a cationic charge in the lowered pH of late endosomes to induce endosomal escape, because of the tertiary amines in their structure. These LNPs have been used, for example, to deliver RNA interference (RNAi) components, as well as genetic constructs or
10 CRISPR-Cas systems. See, such as, Wilbie et al., *Acc Chem Res.*;52(6):1555–1564, 2019. Wang et al., *Proc Natl Acad Sci U S A.*;113(11):2868–2873, 2016 describe use of biodegradable cationic LNPs. Chang et al., *Acc. Chem. Res.*, 52, 665–675, 2019 describe use of ionizable lipid along with cholesterol, DSPC, and a PEGylated lipid to create LNPs.

[00200] Polymer based particles can be used for genetic construct delivery in a similar manner
15 as lipids. Numerous materials have been used for delivery of nucleic acids. For example, cationic polymers such as polyethylenimine (PEI) can be complexed to nucleic acids and can induce endosomal uptake and release, similarly to cationic lipids. Dendrimeric structures of poly(amido-amine) (PAMAM) can also be used for transfection. These particles consist of a core from which the polymer branches. They exhibit cationic primary amines on their surface, which can complex
20 to nucleic acids. Networks based on zinc to aid cross-linking of imidazole have been used as delivery methods, relying on the low pH of late endosomes which, upon uptake, results in cationic charges due to dissolution of the zeolitic imidazole frameworks (ZIF), after which the components are released into the cytosol. Colloidal gold nanoparticles have also been used. See Wilbie et al., *supra*.

[00201] The nuclease can be delivered into a cell in the form of protein or as a nucleic acid
25 encoding the nuclease. Such nucleic acid can be DNA (e.g., circular or linearized plasmid DNA or PCR products) or RNA. For embodiments in which the nuclease coding sequence is delivered in DNA form, it should be operably linked to a promoter to facilitate transcription of the nuclease gene. Mammalian promoters suitable for the invention include constitutive promoters such as the
30 cytomegalovirus early (CMV) promoter (Thomsen et al. (1984). *Proc Natl Acad Sci USA*.

81(3):659-63) or the SV40 early promoter (Benoist and Chambon (1981). Nature. 290(5804):304-10) as well as inducible promoters such as the tetracycline-inducible promoter (Dingermann et al. (1992), Mol Cell Biol. 12(9):4038-45). mRNA encoding the nuclease can be delivered to the cell to reduce the likelihood that the gene encoding the nuclease integrate into the genome of the cell. Such mRNA can be produced using methods known in the art such as in vitro transcription. In some embodiments, the mRNA is capped using 7-methyl-guanosine. In some embodiments, the mRNA may be polyadenylated.

[00202] In certain embodiments, CRISPR-Cas system including a guide nucleic acid and a Cas protein can be combined into a RNP complex and then delivered into the cell as a pre-formed complex. A “ribonucleoprotein” or “RNP,” as used herein, refers to a complex comprising a nucleoprotein and a ribonucleic acid. A “nucleoprotein” as provided herein refers to a protein capable of binding a nucleic acid (e.g., RNA, DNA). Where the nucleoprotein binds a ribonucleic acid it is referred to as “ribonucleoprotein.” The interaction between the ribonucleoprotein and the ribonucleic acid may be direct, e.g., by covalent bond, or indirect, e.g., by non-covalent bond (e.g. electrostatic interactions (e.g. ionic bond, hydrogen bond, halogen bond), van der Waals interactions (e.g. dipole-dipole, dipole-induced dipole, London dispersion), ring stacking (pi effects), hydrophobic interactions, and the like). In certain embodiments, the ribonucleoprotein includes an RNA-binding motif non-covalently bound to the ribonucleic acid. For example, positively charged aromatic amino acid residues (e.g., lysine residues) in the RNA-binding motif may form electrostatic interactions with the negative nucleic acid phosphate backbones of the RNA.

[00203] A variety of delivery methods can be used to introduce an RNP disclosed herein into a cell. Exemplary delivery methods or vehicles include but are not limited to microinjection, liposomes (see, e.g., U.S. Patent Publication No. 2017/0107539) such as molecular trojan horses liposomes that delivers molecules across the blood brain barrier (see, Pardridge et al. (2010) COLD SPRING HARB. PROLOG., doi: 10.1101/pdb.prot54Q7), immunoliposomes, virosomes, microvesicles (e.g. exosomes and ARMMs), polycations, lipidnucleic acid conjugates, electroporation, cell permeable peptides (see, U.S. Patent Publication No. 2018/0363009), nanoparticles, nanowires (see, Shalek et al. (2012) NANO LETTERS, 12: 6498), exosomes, and perturbation of cell membrane (e.g., by passing cells through a constriction in a microfluidic system, see, U.S. Patent Publication No. 2018/0003696), Where the target cell is a proliferating

cell, the efficiency of RNP delivery can be enhanced by cell cycle synchronization (see, U.S. Patent Publication No. 2018/0044700).

[00204] In other embodiments, the dual guide CRISPR-Cas system is delivered into a cell in a “Cas RNA” approach, i.e., delivering a targeter nucleic acid, a modulator nucleic acid, and an RNA (e.g., messenger RNA (mRNA)) encoding a Cas protein. The RNA encoding the Cas protein can be translated in the cell and form a complex with guide nucleic acid intracellularly. Similar to the RNP approach, RNAs have limited half-lives in cells, even though stability-increasing modification(s) can be made in one or more of the RNAs. Accordingly, the “Cas RNA” approach is suitable for active modification of the genetic or epigenetic information in a cell during a limited time period, such as DNA cleavage, and has the advantage of reducing off-targeting. The mRNA can be produced by transcription of a DNA comprising a regulatory element operably linked to a Cas coding sequence.

[00205] A variety of delivery systems can be used to introduce an “Cas RNA” system into a cell. Non-limiting examples of delivery methods or vehicles include microinjection, biolistic particles, liposomes (see, e.g., U.S. Patent Publication No. 2017/0107539) such as molecular trojan horses liposomes that delivers molecules across the blood brain barrier (see, Pardridge *et al.* (2010) COLD SPRING HARB. PROTOC., doi:10.1101/pdb.prot5407), immunoliposomes, virosomes, polycations, lipid:nucleic acid conjugates, electroporation, nanoparticles, nanowires (see, Shalek *et al.* (2012) NANO LETTERS, 12: 6498), exosomes, and perturbation of cell membrane (e.g., by passing cells through a constriction in a microfluidic system, see, U.S. Patent Publication No. 2018/0003696). Specific examples of the “nucleic acid only” approach by electroporation are described in International (PCX) Publication No. WO2016/164356.

[00206] In other embodiments, the CRISPR-Cas system is delivered into a cell in the form of a guide nucleic acid, and a DNA comprising a “regulatory” element operably linked to a Cas coding sequence. As used in this context, the term “operably linked” is intended to mean that the nucleotide sequence of interest is linked to the regulatory element in a manner that allows for expression of the nucleotide sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term “regulatory” element,” as used herein, refers to a transcriptional and/or translational control sequence, such as a promoter, enhancer, transcription termination signal (e.g., polyadenylation signal), internal ribosomal entry

sites (IRES), protein degradation signal, and the like, that provide for and/or regulate transcription of a non-coding sequence (e.g., a targeter nucleic acid or a modulator nucleic acid) or a coding sequence (e.g., a Cas protein) and/or regulate translation of an encoded polypeptide. Such “regulatory” elements are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY, 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., immune cell and nobtaly T cell-specific regulatory sequences). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In certain embodiments, a vector comprises one or more pol III promoter (e.g., 1, 2, 3, 4, 5, or more pol III promoters), one or more pol II promoters (e.g., 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g., 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and HI promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer), the SV40 promoter, the dihydrofolate reductase promoter, the b-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 α promoter. Also encompassed by the term “regulatory element” are enhancer elements, such as WPRE; CMV enhancers; the R-U5' segment in LTR of HTLV-I (see, Takebe et al. (1988) MOL. CELL. BIOL., 8: 466); SV40 enhancer; and the intron sequence between exons 2 and 3 of rabbit b-globin (see, O'Hare et al. (1981) PROG. NATL. ACAD. Set. USA., 78: 1527). It will be appreciated by those skilled in the art that the design of the expression vector can depend on factors such as the choice of the host cell to be transformed, the level of expression desired, etc.

Cell preparation

[00207] Isolation of the cells includes one or more preparation and/or non-affinity based cell separation steps according to well-known techniques in the field. In some examples, cells are washed, centrifuged, and/or incubated in the presence of one or more reagents, for example, to remove unwanted components, enrich for desired components, lyse or remove cells sensitive to

particular reagents. In some examples, cells are separated based on one or more property, such as density, adherent properties, size, sensitivity and/or resistance to particular components.

[00208] In some embodiments, the cell preparation includes steps for freezing, e.g., cryopreserving, the cells, either before or after isolation, incubation, and/or engineering. Any of a variety of known freezing solutions and parameters in some aspects may be used.

[00209] The incubation steps can comprise culture, incubation, stimulation, activation, expansion and/or propagation.

[00210] In some embodiments, the compositions or cells are incubated in the presence of stimulating conditions or a stimulatory agent. Such conditions include those designed to induce proliferation, expansion, activation, and/or survival of cells in the population, to mimic antigen exposure, and/or to prime the cells for genetic engineering, such as for the introduction of an antigen-specific receptor.

[00211] The incubation conditions can include one or more of particular media, temperature, oxygen content, carbon dioxide content, time, agents, e.g., nutrients, amino acids, antibiotics, ions, and/or stimulatory factors, such as cytokines, chemokines, antigens, binding partners, fusion proteins, recombinant soluble receptors, and any other agents designed to activate the cells.

[00212] In some embodiments, the stimulating conditions or agents include one or more agent, e.g., ligand, which is capable of activating an intracellular signaling domain of a TCR complex. In some aspects, the agent turns on or initiates TCR/CD3 intracellular signaling cascade in a T cell. Such agents can include antibodies, such as those specific for a TCR component and/or costimulatory receptor, e.g., anti-CD3, anti-CD28, for example, bound to solid support such as a bead, and/or one or more cytokines. Optionally, the expansion method may further comprise the step of adding anti-CD3 and/or anti CD28 antibody to the culture medium (e.g., at a concentration of at least about 0.5 ng/ml). In some embodiments, the stimulating agents include IL-2 and/or IL-15, for example, an IL-2 concentration of at least about 10 units/mL.

[00213] In some aspects, incubation is carried out in accordance with techniques such as those described in US Patent No. 6,040,177 to Riddell et al., Klebanoff et al., J Immunother. 2012; 35(9): 651-660, Terakura et al., Blood. 2012; 1:72-82, and/or Wang et al. J Immunother. 2012,35(9):689-701.

[00214] In some embodiments, the T cells are expanded by adding to the culture-initiating composition feeder cells, such as non-dividing peripheral blood mononuclear cells (PBMC), (e.g., such that the resulting population of cells contains at least about 5, 10, 20, or 40 or more PBMC feeder cells for each T lymphocyte in the initial population to be expanded); and incubating the culture (e.g. for a time sufficient to expand the numbers of T cells). In some aspects, the non-dividing feeder cells can comprise gamma- irradiated PBMC feeder cells. In some embodiments, the PBMC are irradiated with gamma rays in the range of about 3000 to 3600 rads to prevent cell division. In some aspects, the feeder cells are added to culture medium prior to the addition of the populations of T cells.

[00215] In some embodiments, the stimulating conditions include temperature suitable for the growth of human T lymphocytes, for example, at least about 25 degrees Celsius, generally at least about 30 degrees, and generally at or about 37 degrees Celsius. Optionally, the incubation may further comprise adding non-dividing EBV-transformed lymphoblastoid cells (LCL) as feeder cells. LCL can be irradiated with gamma rays in the range of about 6000 to 10,000 rads. The LCL feeder cells in some aspects is provided in any suitable amount, such as a ratio of LCL feeder cells to initial T lymphocytes of at least about 10:1.

[00216] In embodiments, antigen-specific T cells, such as antigen- specific CD4+ and/or CD8+ T cells, are obtained by stimulating naive or antigen specific T lymphocytes with antigen. For example, antigen-specific T cell lines or clones can be generated to cytomegalovirus antigens by isolating T cells from infected subjects and stimulating the cells in vitro with the same antigen.

[00217] In some aspects, the methods include assessing expression of one or more markers on the surface of the engineered cells or cells being engineered. In one embodiment, the methods include assessing surface expression of one or more target antigen (e.g., antigen recognized by the antigen-specific receptor) sought to be targeted by the adoptive cell therapy, for example, by affinity-based detection methods such as by flow cytometry.

Composition of the invention

[00218] The present invention also includes compositions containing the cells as described herein and/or produced by the provided methods. Typically, said compositions are pharmaceutical compositions and formulations for administration, preferably sterile compositions and formulations, such as for adoptive cell therapy.

[00219] A pharmaceutical composition of the invention generally comprises at least one engineered immune cell of the invention and a sterile pharmaceutically acceptable carrier.

[00220] As used herein the language "pharmaceutically acceptable carrier" includes saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds can further be incorporated into the compositions. In some aspects, the choice of carrier in the pharmaceutical composition is determined in part by the particular engineered CAR or TCR, vector, or cells expressing the CAR or TCR, as well as by the particular method used to administer the vector or host cells expressing the CAR. Accordingly, there are a variety of suitable formulations. For example, the pharmaceutical composition can contain preservatives. Suitable preservatives may include, for example, methylparaben, propylparaben, sodium benzoate, and benzalkonium chloride. In some aspects, a mixture of two or more preservatives is used. The preservative or mixtures thereof are typically present in an amount of about 0.0001 to about 2% by weight of the total composition.

[00221] A pharmaceutical composition is formulated to be compatible with its intended route of administration.

Method of uses and therapeutic applications

[00222] The cells of the disclosure may be used in adoptive cell therapy (notably adoptive T cell therapy or adoptive NK cell therapy). In some embodiments, the use is in the treatment of cancer in a subject in need thereof, but uses also include the treatment of infectious diseases and autoimmune, inflammatory or allergic diseases. In some embodiments, the subject is suffering from a cancer or at risk of suffering from a cancer.

[00223] The cells of the disclosure are particularly beneficial when administered to treat a chronic disease, such as a chronic infectious disease, or when administered to treat refractory, relapsed or resistant cancer.

[00224] In some embodiments, the patient exhibits a cancer relapse or is likely to exhibit a cancer relapse. In some embodiments, the patient exhibits cancer metastasis or is likely to exhibit cancer metastasis. In some embodiments, the patient has not achieved sustained cancer remission

after one or more prior cancer therapies. In some embodiments, the patient suffers from a cancer that is resistant or nonresponsive to one or more prior cancer therapies. In some embodiments, the patient suffers from a refractory cancer. In some embodiments, the patient is likely to exhibit a response to cell therapy that is not durable. In some embodiments, the patient is ineligible for immune checkpoint therapy or did not respond to immune checkpoint therapy. In some embodiments, the patient is ineligible for treatment with high dose of chemotherapy and/or is ineligible for treatment with high adoptive cell therapy doses.

[00225] In such methods, one or more types of modified immune cells as described herein are administered to a subject in need thereof, in an amount effective to treat the disease or disorder. For example, cells expressing one or more antigen-specific receptors as described herein (including with reduced SUV39H1 activity and optionally comprising a CAR with a single active ITAM as described herein) are administered at a dose effective to treat the disease or disorder associated with the antigen(s). Treatment of any of the diseases listed above under the “Antigen” section is contemplated.

[00226] In certain embodiments, immune cells comprising a modified TCR disclosed herein can be used to treat a subject having tumor cells with a low expression level of a surface antigen, .e.g, from a relapse of a disease, wherein the subject received treatment which leads to residual tumor cells. In certain embodiments, the tumor cells have low density of a target molecule on the surface of the tumor cells. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 5,000 molecules per cell, less than about 4,000 molecules per cell, less than about 3,000 molecules per cell, less than about 2,000 molecules per cell, less than about 1,500 molecules per cell, less than about 1,000 molecules per cell, less than about 500 molecules per cell, less than about 200 molecules per cell, or less than about 100 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 2,000 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 1,500 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 1,000 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of between about 4,000 molecules per cell and about 2,000 molecules per cell, between about 2,000 molecules per cell and about 1,000 molecules per cell, between about 1,500 molecules per cell and about 1,000 molecules per cell,

between about 2,000 molecules per cell and about 500 molecules per cell, between about 1,000 molecules per cell and about 200 molecules per cell, or between about 1,000 molecules per cell and about 100 molecules per cell. In certain embodiments, immunoresponsive cells comprising a HI-TCR disclosed herein can be used to treat a subject having a relapse of a disease, wherein the subject received immunoresponsive cells (e.g., T cells) comprising a CAR comprising an intracellular signaling domain that comprises a co-stimulatory signaling domain comprising a 4-1BB polypeptide (e.g., a 4-1BBz CAR). In certain embodiments, the tumor cells have a low density of a tumor specific antigen on the surface of the tumor cells. In certain embodiments, the disease is CD19+ ALL. In certain embodiments, the tumor cells have a low density of CD 19 on the tumor cells. The method of treatment, or application of the present invention is particularly well suited in patient for whom the targeted antigen is expressed at a low density in at least 50 % of the tumor cells, notably 50 % of the tumor cells expressing the said antigen.

[00227] The cells may be administered at certain doses. For example, the immune cells (e.g., T cells or NK cells) in which SUV39H1 has been inhibited may be administered to adults at doses of less than about 10^8 cells, less than about 5×10^7 cells, less than about 10^7 cells, less than about 5×10^6 cells, less than about 10^6 cells, less than about 5×10^5 cells or less than about 10^5 cells. The dose for pediatric patients may be about 100-fold less. In alternative embodiments, any of the immune cells (e.g. T-cells) described herein may be administered to patients at doses ranging from about 10^5 to about 10^9 cells, or about 10^5 to about 10^8 cells, or about 10^5 to about 10^7 cells, or about 10^6 to about 10^8 cells.

[00228] The subject (i.e. patient) is a mammal, typically a primate, such as a human. In some embodiments, the primate is a monkey or an ape. The subject can be male or female and can be any suitable age, including infant, juvenile, adolescent, adult, and geriatric subjects. In some embodiments, the subject is a non-primate mammal, such as a rodent. In some examples, the patient or subject is a validated animal model for disease, adoptive cell therapy, and/or for assessing toxic outcomes such as cytokine release syndrome (CRS). In some embodiments, said subject has a cancer, is at risk of having a cancer, or is in remission of a cancer.

[00229] In some embodiments, the cells or compositions are administered to the subject, such as a subject having or at risk for a cancer or any one of the diseases as mentioned above. In some aspects, the methods thereby treat, e.g., ameliorate one or more symptom of, the disease or

condition, such as with reference to cancer, by lessening tumor burden in a cancer expressing an antigen recognized by the engineered cell.

[00230] Methods for administration of cells for adoptive cell therapy are known and may be used in connection with the provided methods and compositions. For example, adoptive T cell therapy methods are described, e.g., in US Patent Application Publication No. 2003/0170238 to Gruenberg et al; US Patent No. 4,690,915 to Rosenberg; Rosenberg (2011) Nat Rev Clin Oncol. 8(10):577-85). See, e.g., Themeli et al. (2013) Nat Biotechnol. 31 (10): 928-933; Tsukahara et al. (2013) Biochem Biophys Res Commun 438(1): 84-9; Davila et al. (2013) PLoS ONE 8(4): e61338.

[00231] Administration of at least one cell according to the disclosure to a subject in need thereof may be combined with one or more additional therapeutic agents or in connection with another therapeutic intervention, either simultaneously or sequentially in any order. In some contexts, the cells are co-administered with another therapy sufficiently close in time such that the cell populations enhance the effect of one or more additional therapeutic agents, or vice versa. In some embodiments, the cell populations are administered prior to the one or more additional therapeutic agents. In some embodiments, the cell populations are administered after to the one or more additional therapeutic agents.

[00232] With reference to cancer treatment, a combined cancer treatment can include but is not limited to cancer chemotherapeutic agents, cancer vaccination, cytotoxic agents, hormones, anti-angiogens, radiolabelled compounds, immunotherapy, surgery, cryotherapy, and/or radiotherapy.

[00233] In some embodiment a composition comprising an engineered immune cell as herein described is administered in combination with a vaccination regimen. In some embodiment, the cell comprises an antigen receptor (a CAR or a TCR) wherein the antigen is included in the vaccine composition to be administered in the vaccination regimen.

[00234] Conventional cancer chemotherapeutic agents include alkylating agents, antimetabolites, anthracyclines, topoisomerase inhibitors, microtubule inhibitors and B- raf enzyme inhibitors.

[00235] Alkylating agents include the nitrogen mustards (such as mechlorethamine, cyclophosphamide, ifosfamide, melphalan and chlorambucil), ethylenamine and methylenamine

derivatives (such as altretamine, thiotepa), alkyl sulfonates (such as busulfan), nitrosoureas (such as carmustine, lomustine, estramustine), triazenes (such as dacarbazine, procarbazine, temozolomide), and platinum-containing antineoplastic agents (such as cisplatin, carboplatin, oxaliplatin).

5 [00236] Antimetabolites include 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), Capecitabine (Xeloda®), Cytarabine (Ara-C®), Floxuridine, Fludarabine, Gemcitabine (Gemzar®), Hydroxyurea, Methotrexate, Pemetrexed (Alimta®).

[00237] Anthracyclines include Daunorubicin, Doxorubicin (Adriamycin®), Epirubicin, Idarubicin. Other anti-tumor antibiotics include Actinomycin-D, Bleomycin, Mitomycin-C,
10 Mitoxantrone.

[00238] Topoisomerase inhibitors include Topotecan, Irinotecan (CPT-11), Etoposide (VP-16), Teniposide or Mitoxantrone.

[00239] Microtubule inhibitors include Estramustine, Ixabepilone, the taxanes (such as Paclitaxel, Docetaxel and Cabazitaxel), and the vinca alkaloids (such as Vinblastine, Vincristine,
15 Vinorelbine, Vindesine and Vinflunine)

[00240] B-raf enzyme inhibitors include vemurafenib (Zelboraf), dabrafenib (Tafinlar), and encorafenib (Braftovi).

[00241] Immunotherapy includes but is not limited to immune checkpoint modulators (i.e. inhibitors and/or agonists), cytokines, immunomodulating monoclonal antibodies, cancer
20 vaccines.

[00242] Preferably, administration of cells in an adoptive T cell therapy according to the disclosure is combined with administration of immune checkpoint modulators. Examples include inhibitors of (e.g. antibodies that bind specifically to and inhibit activity of) PD-1, CTLA4, LAG
3, BTLA, OX2R, TIM-3, TIGIT, LAIR-1, PGE2 receptors, and/or EP2/4 Adenosine receptors
25 including A2AR. Preferably, the immune checkpoint modulators comprise anti-PD-1 and/or anti-PDL-1 inhibitors (e.g., anti-PD-1 and/or anti-PDL-1 antibodies).

[00243] The present disclosure also relates to the use of a composition comprising the cells as herein described for the manufacture of a medicament for treating a cancer, an infectious disease

or condition, an autoimmune disease or condition, or an inflammatory disease or condition in a subject.

EXAMPLES

Example 1: Targeting designs of a CAR to the *SUV39H1* locus

5 **[00244]** Two ways to disrupt the SUV39H1 locus while simultaneously inserting the CD19-targeting CD28-based second-generation CAR were designed. In the first approach, gRNAs targeting exons 2 and 3 of SUV39H1 (Fig. 1a) and an adeno-associated virus (AAV) vector repair matrix of a left and right homology arms, which encodes a splice-acceptor, a self-cleaving P2A peptide followed by the CAR cDNA and a poly-A tail (Fig. 1b) were designed. In the presence
10 of Cas9 ribonucleoparticles and the AAV repair matrix, the homology driven repair system will incorporate the cassette into the SUV39H1 locus, putting it under the control of the endogenous promoter and simultaneously abrogating the expression of a functional SUV39H1.

15 **[00245]** In the second approach, gRNAs targeting exons 2 and 3 of SUV39H1 (Fig. 1a) and an adeno-associated virus (AAV) vector repair matrix of a left and right homology arms, which encodes in an anti-parallel fashion a complete expression cassette, comprising a promoter (EF1a)
the CAR cDNA and a poly-A tail (Fig. 1c) were designed. In the presence of Cas9 ribonucleoparticles and the AAV repair matrix, the homology driven repair system will incorporate the cassette into the SUV39H1 locus. In this case, while the expression of a functional SUV39H1 is abrogated, the CAR expression cassette is active under its own promoter.

20 **Example 2: Generation of SUV39H1-KO CAR-KI T cells**

[00246] The antiparallel design mentioned above (Fig. 1c) was used to generate T cells knocked out for SUV39H1 and knocked in for CAR (Fig. 2a). Briefly, T cell purification by PBMCs, and activation by aCD3/aCD28 beads and cytokines (IL-7 and IL-15) was performed on day 0. Nucleofection of Cas9 ribonucleoparticles (RNP) carrying was performed on day 3,
25 followed a few hours later infection with AAV particles carrying the repair matrix and expression cassette at different multiplicities of infection. On day 7, the cells were harvested, and then analysed for expression of CAR, SUV39H1, and the memory marker CD27, as well as the trimethylation of residue K9 of Histone 3 (H3K9).

[00247] It was first measured whether expression of SUV39H1 was affected by the CAR cassette incorporation. Fig. 2b shows that, in the presence of the AAV cassette alone, expression of SUV39H1 is unaffected (Fig. 2b, left). However, in the presence of the SUV39H1 targeting gRNA, expression of SUV39H1 is strongly decreased, regardless the presence of the AAV cassette (Fig. 2b right). This was further confirmed by looking into the downstream function of SUV39H1, the levels of H3K9 tri-methylation. Similarly, a decrease in H3K9 tri-methylation levels was detected only in the presence of the SUV39H1 targeting gRNA and regardless the presence of the AAV cassette (Fig. 2c). The expression of a memory marker CD27, known to be targeted for silencing in T cells by SUV39H1 was also investigated. Fig. 2d shows that CD27 expression levels were increased in the presence of the SUV39H1 targeting gRNA and regardless the presence of the AAV cassette. Therefore, the results show that SUV39H1 can be efficiently knocked out by Crispr/Cas9.

[00248] To detect the insertion into the SUV39H1 locus, the CAR expression next assayed. This was performed by flow cytometry. A clear increase in CAR⁺ cell percentage as well as expression levels was detected in T cells treated with different multiplicities of AAV infection and only in the presence of the SUV39H1-targeting gRNA (gRNA SUV vs. Mock, Fig. 2b, c). Therefore, the results demonstrate that a CAR can successfully be inserted into the SUV39H1 locus, and be expressed at the cell surface while inactivating SUV39H1 expression.

CLAIMS

1. An isolated genetically modified immune cell comprising a transgene integrated at a Suv39H1 gene, wherein the transgene comprises a nucleic acid encoding a therapeutic protein and wherein integration of the said transgene at the said Suv39h1 gene disrupts the Suv39H1 gene.
5
2. An isolated genetically modified immune cell comprising in its genome a modified Suv39H1 gene region, wherein said modified Suv39H1 region gene comprises from 5' to 3':
 - (a) a 5' region of said human Suv39h1 region gene;
 - 10 (b) an exogenous polynucleotide; and
 - (c) a 3' region of the human Suv39h1 region gene;wherein the SUV39H1 gene is disrupted.
3. An isolated genetically modified immune cell according to claim 1 or 2, wherein the cell has reduced expression of a SUV39H1 protein, notably reduced expression of a functional Suv39h1 protein when compared to an unmodified control cell.
15
4. An isolated engineered immune cell defective for Suv39H1 and which further comprises a first exogenous nucleic acid encoding a therapeutic protein, wherein said first exogenous nucleic acid is inserted in the Suv39h1 gene region and wherein said insertion in the Suv39h1 gene region disrupts the SUV39H1 gene.
- 20 5. An isolated genetically modified immune cell according to any one of claims 1-4, wherein the transgene or exogenous nucleic acid or polynucleotide is inserted in an intron or an exon of the SUV39H1 gene.
6. An isolated genetically modified immune cell according to any one of claims 1-5, which is defective for at least one more gene selected from full list preferably a SOCS1, FAS, TRAC, TRBC, TRGC, TRDC, CD3 Delta, CD3 Epsilon, CD3 Gamma, CD3 Zeta (CD247), B2M, CD4, CD8 alpha, CD8 beta, CTLA4, PD-1, TIM-3, LAG3, TIGIT, CD28, CD25, CD69, CD95 (Fas), CD52, CD56, CD38, KLRG-1, SOCS1, CIITA, HLA-E and NK specific genes (e.g, NKG2A, NKG2C, NKG2D, NKp46), CD 16, CD84,
25

CD84, 2B4, and KIR-L, preferably SOCS1, FAS, TRAC, TRBC, B2M, CIITA, preferably SOCS1, TRAC, B2M and/or FAS.

7. A method for producing a genetically modified immune cell comprising a modified Suv39H1 region gene, said method comprising: introducing into an immune cell

- (i) a first nucleic acid sequence comprising a first exogenous polynucleotide encoding an engineered nuclease protein, or (ii) an engineered nuclease protein, wherein said engineered nuclease produces a cleavage site at a recognition sequence within said Suv39h1 region gene; and

- a second nucleic acid sequence comprising a second exogenous polynucleotide encoding a therapeutic protein.

8. A method for producing a genetically modified immune cell comprising a modified human Suv39H1 region gene, said method comprising:

a) introducing into a cell:

- a first nucleic acid sequence encoding an engineered nuclease; or
- an engineered nuclease protein;

wherein said engineered nuclease produces a cleavage site at a recognition sequence within said human Suv39h1 region gene; and

b) introducing into said cell a second nucleic acid sequence comprising an exogenous polynucleotide;

wherein the sequence of said exogenous polynucleotide is inserted into said Suv39h1 region gene at said cleavage site; and further wherein said genetically-modified immune cell has reduced expression of a functional Suv39H1 protein when compared to an unmodified control immune cell.

9. An isolated genetically modified immune cell according to any one of claims 1-6 or a method according to claim 7 or 8, wherein the therapeutic protein is intracellular or surface membrane protein notably a CAR an exogenous TCR including modified TCR.

10. A modified immune cell according to claim 9, wherein the antigen is orphan tyrosine kinase receptor ROR1, tEGFR, Her2, p95HER2, LI-CAM, CD19, CD20, CD22, mesothelin, CEA, Claudin 18.2, hepatitis B surface antigen, anti-folate receptor, CD23,

CD24, CD30, CD33, CD38, CD44, EGFR, EGP-2, EGP-4, CD70, EPHA2, ErbB2, 3, or 4, FcRH5, FBP, fetal acetylcholine receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-13R-alpha2, kdr, kappa light chain, BCMA, Lewis Y, MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, TAG72, VEGF-R2, carcinoembryonic antigen (CEA), prostate specific antigen (PSMA), estrogen receptor, progesterone receptor, ephrinB2, CD 123, CS-1, c-Met, GD-2, MAGE A3, CE7, or Wilms Tumor 1 (WT-1); or optionally any of the tumor neoantigenic peptides disclosed in Int. Pat. Pub. No. WO 2021/043804.

11. A modified immune cell according to any one of claims 1 to 6, 9 or 10, wherein the cancer is a myeloid or a lymphoid cancer.
12. A modified immune cell according to any one of claims 1 to 6, 9 or 10, wherein the cancer is a solid tumor.
13. A modified immune cell according to claim 12, wherein the solid tumor is a cancer affecting an organ, optionally colon, rectum, skin, endometrium, lung (including non-small cell lung carcinoma), uterus, bones (such as Osteosarcoma, Chondrosarcomas, Ewing's sarcoma, Fibrosarcomas, Giant cell tumors, Adamantinomas, and Chordomas), liver, kidney, esophagus, stomach, bladder, pancreas, cervix, brain (such as Meningiomas, Glioblastomas, Lower-Grade Astrocytomas, Oligodendrocytomas, Pituitary Tumors, Schwannomas, and Metastatic brain cancers), ovary, breast (such as triple negative, or luminal breast cancer), head and neck region, testis, prostate or the thyroid gland.
14. A modified immune cell according to any one of claims 1 to 6, 9 or 10, wherein the patient suffers from a chronic viral infection, and optionally wherein the immune cell allows long term infection control and/or increased viral clearance.
15. A modified immune cell according to claim 14, wherein the chronic viral infection is caused by human immunodeficiency virus (HIV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus, BK polyomavirus, hepatitis virus, such as hepatitis B, hepatitis C, hepatitis D, hepatitis E.

16. A modified immune cell according to any one of claims 1 to 6, 9 to 15, wherein the SUV39H1 activity is inhibited.
17. A modified immune cell according to claim 16, which has been contacted with an exogenous SUV39H1 inhibitor, optionally a nucleic acid inhibitor of SUV39H1.
- 5 18. A modified immune cell according to claim 17, wherein the exogenous SUV39H1 inhibitor is (a) a dominant negative inhibitor, or (b) an RNAi, shRNA, ribozyme or antisense oligonucleotide complementary to a fragment of the SUV39H1 gene, or (c) an epipolythiodioxopiperazine (ETP) class of SUV39H1 inhibitor.
- 10 19. A modified immune cell according to any one of claims 1 to 6, 9 to 15, wherein the cell's SUV39H1 gene comprises one or more mutations that results in a deleted or non-functional SUV39H1 protein, or a SUV39H1 protein with reduced activity.
20. A modified immune cell according to any one of claims 9-19, wherein the antigen-specific receptor is a modified TCR.
- 15 21. A modified immune cell according to any one of claims 9-19, wherein the antigen-specific receptor is a chimeric antigen receptor (CAR).
- 20 22. A modified immune cell according to any one of claims 1 to 6, 9 to 21, wherein the cell expresses at least one antigen-specific receptor having an intracellular signaling domain wherein one or two immunoreceptor tyrosine-based activation motifs (ITAMs) are inactivated, optionally wherein the antigen-specific receptor comprises a single active ITAM domain.
- 25 23. A modified immune cell according to any one of claims 9 to 22, wherein the antigen-specific receptor is a chimeric antigen receptor (CAR) comprising:
a) an extracellular antigen-binding domain that specifically binds an antigen,
b) a transmembrane domain,
c) optionally one or more costimulatory domains, and
d) an intracellular signaling domain wherein one or two immunoreceptor tyrosine-based activation motifs (ITAMs) are inactivated, optionally wherein the antigen-

specific receptor comprises a single active ITAM domain, optionally an intracellular domain comprising a modified CD3zeta intracellular signaling domain in which ITAM2 and ITAM3 have been inactivated.

24. A modified immune cell according to any one of claims 9 to 23, wherein the antigen-specific receptor comprises an extracellular antigen-binding domain which is an scFv; or an antibody heavy chain region (VH) and/or an antibody variable region (VL); or optionally a bispecific or trispecific antigen-binding domain.
25. A modified immune cell according to any one of claims 9 to 24, wherein the transmembrane domain is from CD28, CD8 or CD3-zeta, or a fragment thereof.
26. A modified immune cell according to any one of claims 9 to 25, wherein the one or more costimulatory domains are selected from the group consisting of: 4-1BB (CD137), CD28, CD27, ICOS, OX40 (CD134) and DAP10; DAP12, 2B4, CD40, FCER1G and/or GITR (AITR), or an active fragment thereof.
27. A modified immune cell according to any one of claims 9 to 26, wherein the antigen-specific receptor comprises:
- a) an extracellular antigen-binding domain that specifically binds an antigen, optionally comprising an antibody heavy chain variable region and/or an antibody light chain variable region, and is optionally bispecific or trispecific;
 - b) a transmembrane domain, optionally comprising a fragment of transmembrane domain of alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD34, CD137, or CD154, NKG2D, OX40, ICOS, 2B4, DAP10, DAP12, CD40; and
 - c) optionally one or more co-stimulatory domains from 4-1BB, CD28, ICOS, OX40, DAP10 or DAP12, 2B4, CD40, FCER1G, or an active fragment thereof;
 - d) an intracellular signaling domain comprising an intracellular signaling domain from CD3zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CDS, CD22, CD79a, CD79b, or CD66d, 2B4, or an active fragment thereof.

28. A modified immune cell according to any one of claims 9 to 27, wherein the antigen-specific receptor is a chimeric antigen receptor (CAR) comprising:
- a) an extracellular antigen-binding domain, optionally an scFv,
 - b) a transmembrane domain, optionally from CD28, CD8 or CD3-zeta,
 - 5 c) one or more co-stimulatory domains, optionally from 4-1 BB, CD28, ICOS, OX40 or DAP10, and
 - d) an intracellular signaling domain from CD3zeta, optionally in which ITAM2 and ITAM3 have been inactivated.
29. A modified immune cell according to any one of claims 9 to 27, wherein the antigen-specific receptor is a modified TCR that comprises a heterologous extracellular antigen-binding domain that specifically binds an antigen, optionally comprising an antibody heavy chain variable region and/or an antibody light chain variable region, and is optionally bispecific or trispecific.
30. A modified immune cell according to any one of claims 9 to 27, wherein the antigen-specific receptor is a modified TCR that comprises a fragment of an alpha, beta, gamma or epsilon chain and a heterologous extracellular antigen-binding domain that specifically binds an antigen, optionally comprising an antibody heavy chain variable region and/or an antibody light chain variable region, optionally an scFv, and is optionally bispecific or trispecific.
31. A modified immune cell according to any one of claims 9 to 27, wherein the antigen-specific receptor is a modified TCR that comprises:
- a) a first antigen-binding chain comprising an antigen-binding fragment of a heavy chain variable region (VH) of an antibody; and
 - b) a second antigen-binding chain comprising an antigen-binding fragment of a light chain variable region (VL) of an antibody;
- wherein the first and second antigen-binding chains each comprise a TRAC polypeptide or a TRBC polypeptide, optionally wherein at least one of the TRAC polypeptide and the TRBC polypeptide is endogenous, and optionally wherein one or both of the endogenous TRAC and TRBC polypeptides is inactivated.

32. A modified immune cell according to any one of claims 1 to 6 and 9 to 31, wherein a heterologous nucleic acid sequence encoding the antigen-specific receptor, or a portion thereof is inserted into the cell genome to express the antigen-specific receptor.
- 5 33. A modified immune cell according to any one of claims 1 to 6 and 9 to 31, wherein a heterologous nucleic acid sequence outside the cell genome expresses the antigen-specific receptor.
34. A modified immune cell according to claim 32, wherein insertion of the heterologous nucleic acid sequence encoding the antigen-specific receptor, or a portion thereof inactivates expression of a native TCR alpha chain and/or a native TCR beta chain.
- 10 35. A modified immune cell according to any of claims 9 to 34, wherein expression of the antigen-specific receptor is under control of an endogenous promoter of a TCR, optionally an endogenous TRAC promoter.
- 15 36. A modified immune cell according to any of claims 1 to 6 and 9 to 35, wherein the cell is a T cell, a CD4⁺ T cell, a CD8⁺ T cell, a CD4⁺ and CD8⁺ T cell, a NK cell, a T regulatory cell, a T_N cell, a memory stem T cell (T_{SCM}), a T_{CM} cell, a T_{EM} cell, a monocyte, a dendritic cell, or a macrophage, or a progenitor thereof, optionally a T cell progenitor, a lymphoid progenitor, an NK cell progenitor, a myeloid progenitor, a pluripotent stem cell, an induced pluripotent stem cell (iPSC), a hematopoietic stem cell (HSC), an adipose derived stem cell (ADSC), or a pluripotent stem cell of myeloid or lymphoid lineage.
- 20 37. A modified immune cell according to claim 36, wherein the immune cell is a T cell or NK cell, or progenitor thereof.
38. A modified immune cell according to any of claims 1 to 6 and 9 to 37, that further comprises a second engineered antigen-specific receptor, optionally a modified TCR or CAR, that specifically binds to a second antigen.
- 25 39. A modified immune cell according to any of claims 1 to 6 and 9 to 37, that comprises a first CAR that binds the antigen and a second CAR that binds a second antigen.

40. A modified immune cell according to any of claims 1 to 6 and 9 to 37, that comprises a CAR that binds the antigen and modified TCR that binds a second antigen.
41. A modified immune cell according to any of claims 1 to 6 and 9 to 37, that comprises a first modified TCR that binds the antigen and a second modified TCR that binds a second antigen.
42. A modified immune cell according to any of claims 1 to 6 and 9 to 37, that comprises three or more engineered antigen-specific receptors.
43. A modified immune cell according to any of claims 1 to 6 and 9 to 42, that further comprises a heterologous co-stimulatory receptor.
44. A modified immune cell according to claim 43, wherein the co-stimulatory receptor comprises (a) an extracellular domain of a co-stimulatory ligand, optionally from CD80, (b) a transmembrane domain, optionally from CD80, and (c) an intracellular domain of a co-stimulatory molecule, optionally CD28, 4-1BB, OX40, ICOS, DAP10, CD27, CD40, NKGD2, or CD2, preferably 4-1BB.
45. A modified immune cell according to any of claims 9 to 44, wherein the extracellular antigen-binding domain binds an antigen with a K_D affinity of about 1×10^{-7} or less, about 5×10^{-8} or less, about 1×10^{-8} or less, about 5×10^{-9} or less, about 1×10^{-9} or less, about 5×10^{-10} or less, about 1×10^{-10} or less, about 5×10^{-11} or less, about 1×10^{-11} or less, about 5×10^{-12} or less, or about 1×10^{-12} or less.
46. A modified immune cell according to any of claims 9 to 45, wherein the antigen has a low density on the cell surface, of less than about 10,000, or less than about 5,000, or less than about 2,000 molecules per cell.
47. A modified immune cell according to any of claims 1 to 6 and 9 to 46, wherein SUV39H1 expression is reduced or inhibited by at least about 50%, 60%, 70%, 75%, 80%, 85%, 90% or 95%.
48. A modified immune cell for use according to any of claims 1 to 6 and 9 to 47, wherein endogenous TCR expression is reduced by at least about 75%, 80%, 85%, 90% or 95%.

49. A modified immune cell for use according to any of claims 1 to 6 and 9 to 49 that is allogeneic.
50. A modified immune cell according to any of claims 1 to 6 and 9 to 49 that is autologous.
51. A modified immune cell according to any of claims 1 to 6 and 9 to 50, wherein the HLA-A locus is inactivated.
52. A modified immune cell according to claim 51, wherein HLA class I expression is reduced by at least about 75%, 80%, 85%, 90% or 95%.
53. A method of producing the cell of any of claims 1 to 6 and 9 to 52 comprising (a) introducing into the cell (a) a SUV39H1 inhibitor, and (b) (i) a nucleic acid encoding a CAR having one active ITAM or (ii) a heterologous nucleic acid encoding a portion of a modified TCR.
54. A modified immune cell according to any of claims 1 to 6 and 9 to 52, wherein the immune cell is a CAR T-cell and a dose of less than about 5×10^7 cells, optionally about 10^5 to about 10^7 cells, is administered to the subject.
55. A modified immune cell according to any of claims 1 to 6, 9 to 52 and 54, wherein a second therapeutic agent, optionally one or more cancer chemotherapeutic agents, cytotoxic agents, cancer vaccines, hormones, anti-angiogens, radiolabelled compounds, immunotherapy, surgery, cryotherapy, and/or radiotherapy, is administered to the subject.
56. A modified immune cell according to any of claims to 1 to 6, 9 to 52, 54-55, wherein the second therapeutic agent is an immune checkpoint modulator.
57. A modified immune cell according to claim 56, wherein the immune checkpoint modulator is an antibody that specifically binds to, or other inhibitor of, PD1, PDL1, CTLA4, LAG3, BTLA, OX2R, TIM-3, TIGIT, LAIR-1, PGE2 receptor, EP2/4 adenosine receptor, or A2AR, optionally an anti-PD1 or anti-PDL1 antibody.

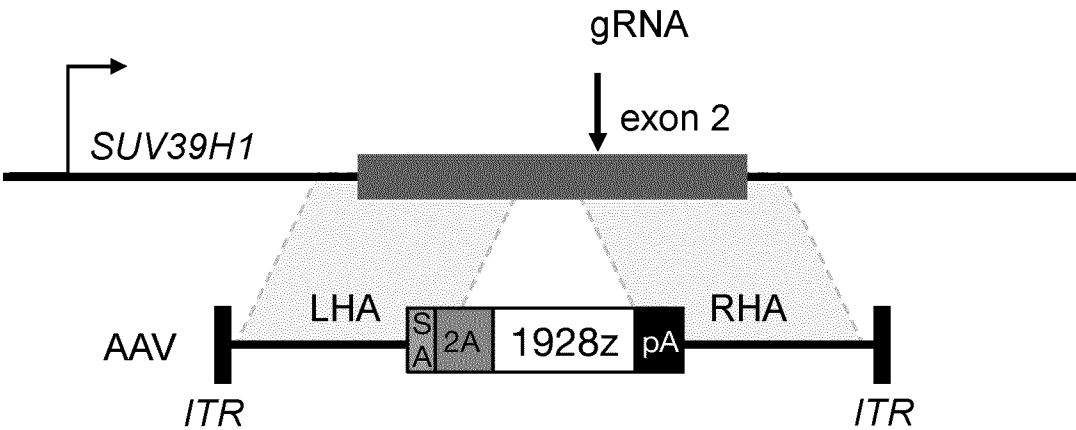
1/4

Fig. 1

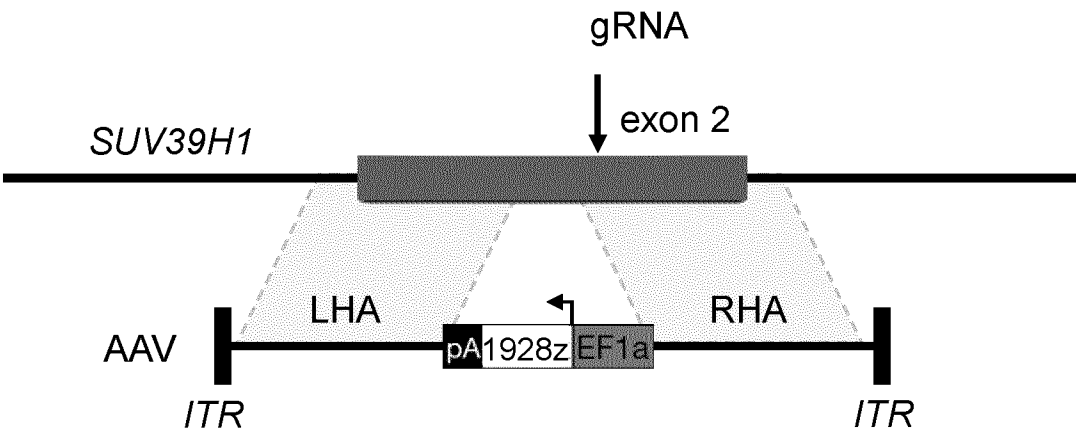
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gRNA name	SUV39H1 Exon
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gRNA-SUV-2	3
gRNA-SUV-3	3
gRNA-SUV-4	3
gRNA-SUV-5	3

b



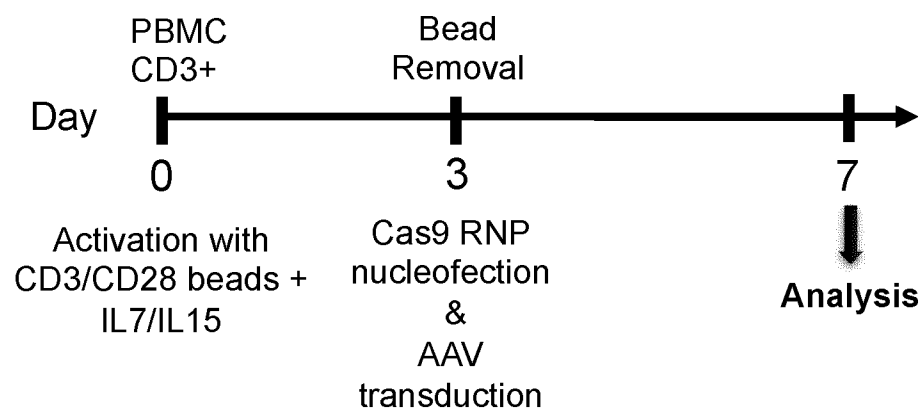
c



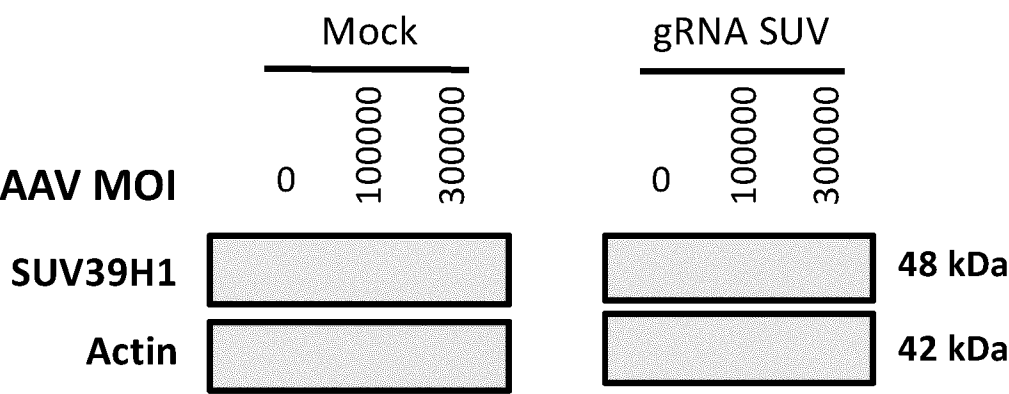
2/4

Fig. 2

a



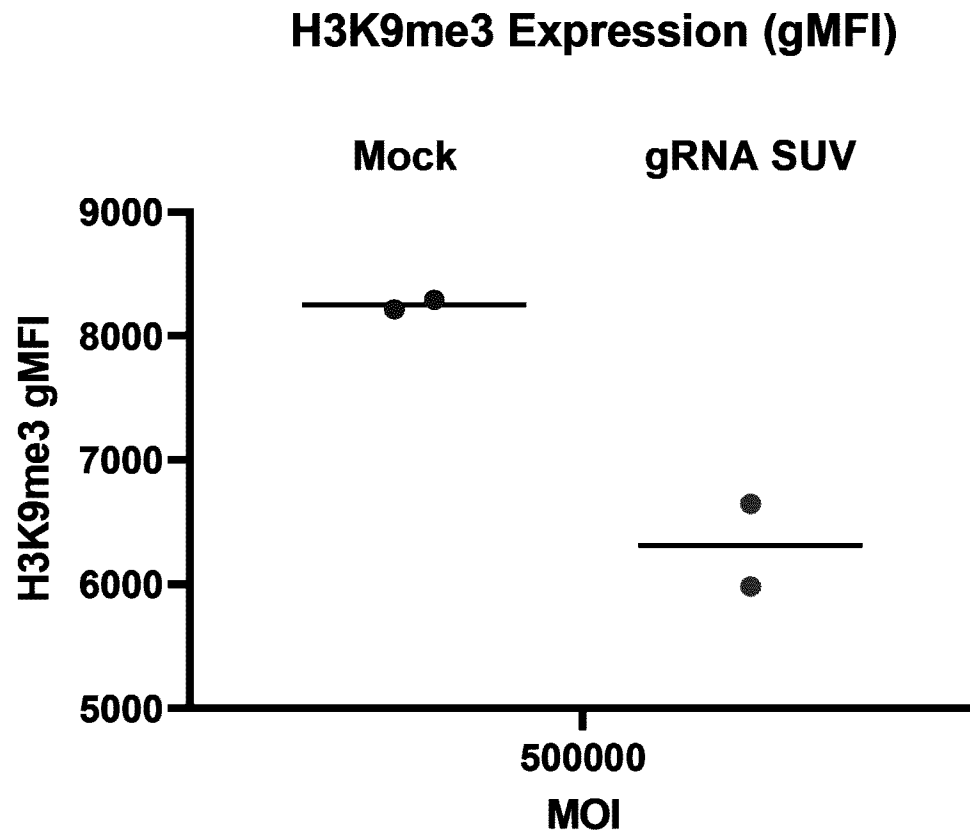
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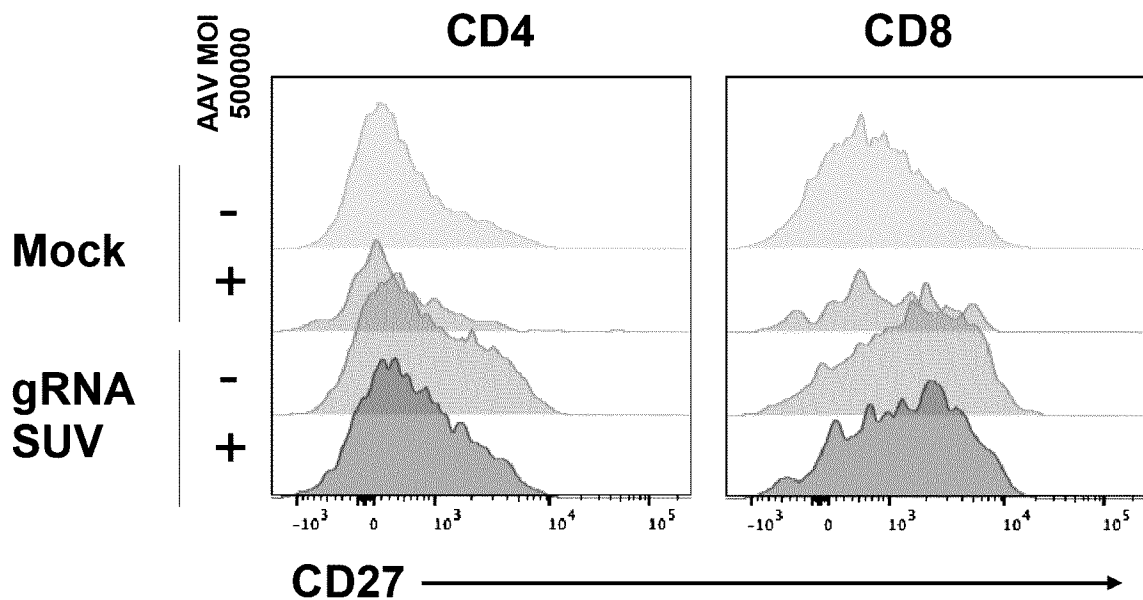
3/4

Fig. 2 (continuation)

C

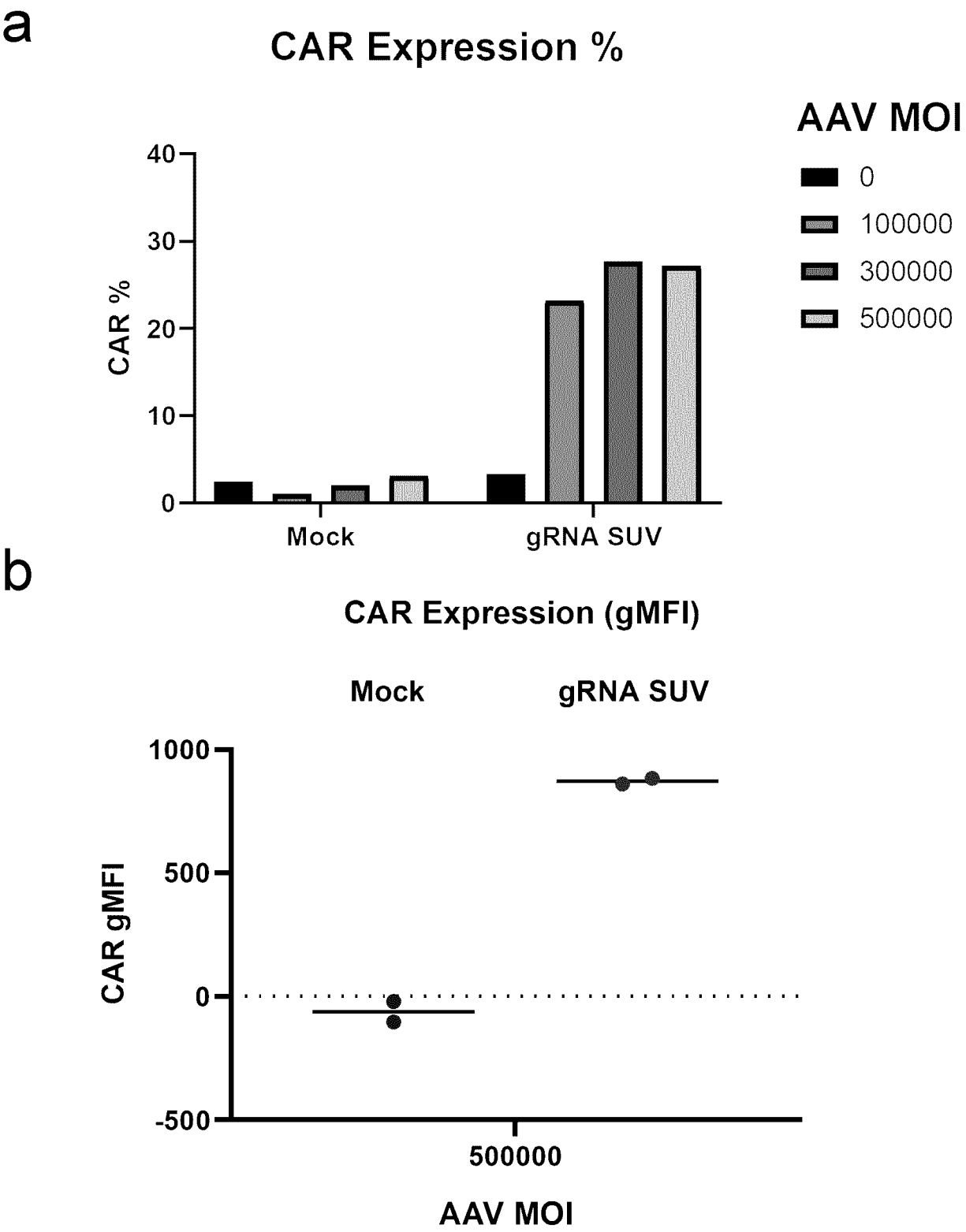
**CD27 Expression**

d



4/4

Fig. 3



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2023/076437

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/00 C12N15/113

ADD.

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)

A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2021/013950 A1 (MNEMO THERAPEUTICS [FR]; INST CURIE [FR]; INST NAT SANTE RECH MED [FR]) 28 January 2021 (2021-01-28) figure 1; example 1 figures 2, 3; example 2 figure 6; example 5 figure 8; example 7 figures 9, 11, 12 figure 16; example 9 figures 19, 20; example 11 figure 21; example 12 example 13 ----- -/--	1-57



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

3 January 2024

Date of mailing of the international search report

26/01/2024

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2023/076437

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Jain Nayan ET AL: "SUV39H1 disruption imparts functional persistence to CD28-costimulated human CAR T cells", Annual Meeting of the American Association of Immunologists, IMMUNOLOGY 2022, 1 May 2022 (2022-05-01), pages 1-2, XP93115369, DOI: https://doi.org/10.4049/jimmunol.208.Supp.122.05 Retrieved from the Internet: URL:https://journals.aai.org/jimmunol/article/208/1_Supplement/122.05/236802/SUV39H1-disruption-imparts-functional-persistence [retrieved on 2024-01-02] the whole document -----	1-57

INTERNATIONAL SEARCH REPORT

International application No.

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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.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2023/076437

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2021013950 A1	28-01-2021	AU 2020318781 A1	10-02-2022
		BR 112022001148 A2	15-03-2022
		CA 3146895 A1	28-01-2021
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