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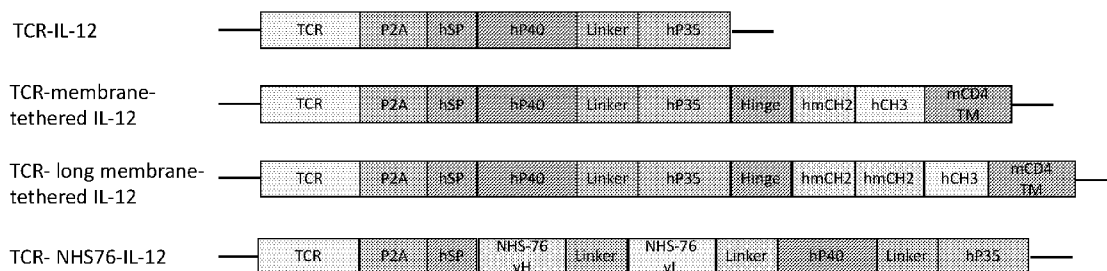


FIG. 1C

(57) Abstract: This disclosure relates to IL-12 armored immune cell therapies and the use of IL-12 in combination with immune cell therapies.

IL-12 ARMORED IMMUNE CELL THERAPY AND USES THEREOF

TECHNICAL FIELD

This disclosure relates to IL-12 armored immune cell therapies and the use of IL-12 in combination with immune cell therapies.

BACKGROUND

Cancer is one of the most widespread cellular anomalies caused by biological and environmental factors, such as age, gender, genetic mutations, environmental exposure such as UV radiation, occupational risk factors, carcinogens, asbestos, radioactive materials, and viral infections (e.g., HPV, EBV, HBV, HCV, HTLV-1 and KSHV) (Margaret E *et al.*, “Viruses Associated With Human Cancer,” *Biochimica et Biophysica Acta*. 1782:127–150 (2008)).

The recent clinical and commercial success of CAR T-cell therapy has created great interest in immune cell therapies. Despite advancement in cancer treatments, the efficacy of various treatments for certain cancers is relatively poor. Accordingly, there exists an unmet need for effective therapies for cancers.

SUMMARY

The present disclosure provides modified (e.g., membrane tethered) or unmodified IL-12, which can be used in combination with immune cell therapies (e.g., TCR-T, CAR-T, or TIL) to treat cancers (e.g., a solid tumor). The present disclosure also provides the use of IL-12 fused with a tumor-targeting antibody (e.g., NHS76) in combination with immune cell therapies (e.g., TCR-T, CAR-T, CAR-NK, or TIL) to treat cancers. The use of the modified IL-12 in combination with immune cell therapies can greatly increase the efficacy of these immune cell therapies; in addition, these methods have improved safety for clinical use.

In one aspect, provided herein is a cell expressing (a) an exogenous T cell receptor (TCR), or a chimeric antigen receptor (CAR); and (b) IL-12.

In some embodiments, the IL-12 is a membrane tethered IL-12.

In some embodiments, the membrane tethered IL-12 comprises a CD4 transmembrane region.

In some embodiments, the membrane tethered IL-12 comprises an immunoglobulin CH2 domain, an immunoglobulin CH3 domain, and a CD4 transmembrane region.

In some embodiments, the membrane tethered IL-12 comprises two or more immunoglobulin CH2 domains, one or more immunoglobulin CH3 domains, and a CD4 transmembrane region.

In some embodiments, the immunoglobulin CH2 domain is a wild-type immunoglobulin constant domain.

In some embodiments, the amino acid at position 235 (EU numbering) of the immunoglobulin CH2 domain is Glu and the amino acid residue at position 297 (EU numbering) of the immunoglobulin CH2 domain is Gln.

In some embodiments, the membrane tethered IL-12 further comprises an immunoglobulin hinge region.

In some embodiments, the immunoglobulin CH2 domain is a human immunoglobulin CH2 domain, the immunoglobulin CH3 domain is a human immunoglobulin CH3 domain, and the CD4 transmembrane region is a human CD4 transmembrane region.

In some embodiments, the IL-12 is a soluble IL-12.

In some embodiments, the IL-12 is linked to a tumor-targeting antibody or antigen-binding fragment thereof.

In some embodiments, the tumor-targeting antibody or antigen binding fragment thereof is a single-chain variable fragment (scFv).

In some embodiments, the tumor-targeting antibody is NHS76.

In some embodiments, the IL-12 is a human IL-12 or a mouse IL-12.

In some embodiments, the TCR or CAR targets BCMA, CD19, CD22, CD30, CD33, CD56, CD123 (IL-3R), CEA, IL13Ra2, ALPP, EBV-related antigens (e.g., LMP2), EGFR, EGFRvIII, GD2, GPC3, HER2, a HPV-related antigen (e.g., E6 or E7), MAGE (e.g., MAGE-A3), Mesothelin, MUC-1, NY-ESO-1, PSCA, PSMA, ROR1, WT1, or Claudin 18.2.

In some embodiments, the CAR comprises an extracellular domain,

In some embodiments, the extracellular domain is a single chain variable fragment (scFv), a ligand (e.g., a receptor-binding ligand), or an antibody mimetic.

In one aspect, provided herein is a vector comprising: a) a first nucleic acid sequence encoding an IL-12 alpha subunit and an IL-12 beta subunit; b) a second nucleic acid sequence

encoding one or more immunoglobulin CH2 domains, one or more immunoglobulin CH3 domains, and a transmembrane region. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a first linker sequence.

In some embodiments, the first linker sequence encodes an immunoglobulin hinge polypeptide sequence.

In some embodiments, the transmembrane region is a CD4 transmembrane region.

In some embodiments, the transmembrane region is an immunoglobulin transmembrane region.

In some embodiments, the IL-12 alpha subunit and the IL-12 beta subunit are linked by a linker peptide sequence.

In some embodiments, the vector further comprises a sequence encoding a signal peptide.

In some embodiments, the IL-12 alpha subunit is a human IL-12 alpha subunit, the IL-12 beta subunit is a human IL-12 beta subunit, the immunoglobulin CH2 domain is a human immunoglobulin CH2 domain, the immunoglobulin CH3 domain is a human immunoglobulin CH3 domain.

In some embodiments, the vector further comprises a third nucleic acid sequence encoding a T cell receptor (TCR), or a chimeric antigen receptor (CAR).

In some embodiments, the third nucleic acid sequence is linked to the first nucleic acid by a second linker sequence. In some embodiments, the second linker sequence encodes a P2A sequence.

In some embodiments, the first nucleic acid and the second nucleic acid are under control of a regulatory element (e.g., a promotor).

In some embodiments, the first nucleic acid, the second nucleic acid, and the third nucleic acid are under control of a regulatory element (e.g., a promotor).

In one aspect, provided herein is a vector comprising: a) a first nucleic acid sequence encoding a heavy chain variable region (VH) and a light chain variable region (VL) of a tumor-targeting antibody; b) a second nucleic acid sequence encoding an IL-12 alpha subunit and an IL-12 beta subunit. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a first linker sequence.

In some embodiments, the tumor-targeting antibody targets a tumor-associated antigen. In some embodiments, the tumor-targeting antibody is NHS76.

In some embodiments, the vector further comprises a nucleic acid encoding a signal peptide.

In some embodiments, the signal peptide is a human signal peptide, the IL-12 alpha subunit is a human IL-12 alpha subunit, and the IL-12 beta subunit is a human IL-12 beta subunit.

In some embodiments, the vector further comprises a third nucleic acid sequence encoding a T cell receptor (TCR), or a chimeric antigen receptor (CAR).

In some embodiments, the third nucleic acid sequence is linked to 5' of the first nucleic acid by a second linker sequence. In some embodiments, the second linker sequence encodes a P2A sequence.

In some embodiments, the first nucleic acid and the second nucleic acid are under control of a regulatory element (e.g., a promotor).

In some embodiments, the first nucleic acid, the second nucleic acid, and the third nucleic acid are under control of a regulatory element (e.g., a promotor).

In one aspect, provided herein is a vector comprising, in a 5' to 3' direction a) a first nucleic acid sequence encoding an exogenous T cell receptor (TCR), or a chimeric antigen receptor (CAR); b) a second nucleic acid sequence encoding a signal peptide, an IL-12 alpha subunit and an IL-12 beta subunit. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a linker sequence.

In some embodiments, the IL-12 alpha subunit and the IL-12 beta subunit are linked by a linker peptide sequence.

In some embodiments, the linker sequence encodes a P2A sequence

In one aspect, provided herein is a fusion polypeptide, comprising a) a first region comprising an IL-12 alpha subunit, and an IL-12 beta subunit. In some embodiments, the IL-12 alpha subunit and the IL-12 beta subunit are linked by a linker peptide sequence. b) a second region comprising one or more immunoglobulin CH2 domains, one or more immunoglobulin CH3 domains, and a transmembrane region.

In some embodiments, the first region and the second region are linked by an immunoglobulin hinge peptide.

In one aspect, provided herein is a fusion polypeptide, comprising a) a first region comprising an IL-12 alpha subunit, and an IL-12 beta subunit. In some embodiments, the IL-12 alpha subunit and the IL-12 beta subunit are linked by a first linker peptide sequence. b) a

second region comprising a heavy chain variable region (VH) and a light chain variable region (VL) of a tumor-targeting antibody. In some embodiments, the VH and the VL are linked by a second linker peptide sequence. In some embodiments, the first region and the second region are linked by a third linker peptide sequence.

In some embodiments, the third linker polypeptide sequence has a sequence that is at least 80% identical to SEQ ID NO: 17.

In one aspect, provided herein is a nucleic acid encoding the polypeptide as described herein.

In one aspect, provided herein is a vector comprising the nucleic acid as described herein.

In one aspect, provided herein is a cell expressing the fusion polypeptide as described herein.

In one aspect, provided herein is a cell comprising the vector as described herein.

In some embodiments, the cell secretes a higher level of a cytokine as compared to a same cell except that the cell does not comprise the vector as described herein. In some embodiments, the cell stimulates one or more cells in the vicinity of the cell to secrete a cytokine. In some embodiments, the cytokine is an IFN γ .

In some embodiments, the cell expresses a higher level of an early TCR activation marker as compared to a same cell except that the cell does not comprise the vector as described herein, and/or stimulates one or more cells in the vicinity of the cell to express the early TCR activation marker.

In some embodiments, the activation marker is a CD69.

In some embodiments, the cell is a cell line.

In some embodiments, the cell is a primary cell obtained from a subject (e.g., a human subject).

In some embodiments, the cell is an immune cell (e.g., a lymphocyte).

In some embodiments, the cell is a tumor-infiltrating lymphocyte (TIL) or a NK cell (e.g., a CAR-NK cell).

In some embodiments, the cell is a T cell. In some embodiments, the T-cell is isolated from a human subject. In some embodiments, the T cell is CD8 $^{+}$. In some embodiments, the T cell is CD4 $^{+}$.

In some embodiments, the vector is an expression vector, a viral vector, a retroviral vector, or a lentiviral vector. In some embodiments, the retroviral vector is pMP71.

In one aspect, provided herein is a method for producing a cell, comprising introducing the vector as described herein into the cell *in vitro* or *ex vivo*. In some embodiments, the vector is introduced into the cell by transduction.

In one aspect, provided herein is a method of treating a subject having a cancer, the method comprising administering to the subject in need thereof, an effective amount of the cell as described herein.

In some embodiments, the cancer is a heterogeneous cancer. In some embodiments, the cancer is a homogeneous cancer.

In some embodiments, the cell is isolated from peripheral blood mononuclear cells (PBMCs) of the subject.

In one aspect, provided herein is a method of treating a subject having a cancer, the method comprising administering to the subject in need thereof, a) an effective amount of cells expressing a T cell receptor (TCR), or a chimeric antigen receptor (CAR); and b) an effective amount of a protein comprising an IL-12 and a tumor-targeting antibody or antigen binding fragment thereof.

In some embodiments, the cell is isolated from peripheral blood mononuclear cells of the subject.

In one aspect, provided herein is a method of treating a human subject having a cancer, the method comprising providing cells collected from the human subject or a different human subject; introducing the vector as described herein in to the cells; culturing and expanding the cells; and administering an effective amount of composition comprising the cells to the subject.

In some embodiments, the cancer is a heterogeneous cancer. In some embodiments, the cancer is a homogeneous cancer.

In some embodiments, the cells are peripheral blood mononuclear cells (PBMC).

In some embodiments, the cells are tumor-infiltrating lymphocytes and the vector comprises a nucleic acid encoding IL-12. In some embodiments, the IL-12 is a membrane tethered IL-12.

In some embodiments, the cells are T cells and the vector comprises a nucleic acid encoding TCR or CAR. In some embodiments, the vector further comprises a nucleic acid encoding IL-12. In some embodiments, the IL-12 is a membrane tethered IL-12.

As used herein, the term “genetically engineered cell” or “genetically modified cell” refers to a cell with a modification of a nucleic acid sequence in the cell, including, but not limited to, a cell having a insertion, deletion, substitution, or modification of one or more nucleotides in its genome, and/or a cell with an exogenous nucleic acid sequence (e.g., a vector), wherein the exogenous nucleic acid sequence is not necessarily integrated into the genome.

As used herein, the term “membrane tethered IL-12” refers to IL-12 in a modified form that is tethered to a cell membrane.

As used herein, the term "peripheral blood cells" refers to cells normally found in the peripheral blood including, but is not limited to, eosinophils, neutrophils, T cells, monocytes, K cells, granulocytes, and B cells.

As used herein, the term “cancer” or “cancer cell” refers to the cells dividing in an uncontrolled manner. Examples of such cells include cells having an abnormal state or condition characterized by rapidly proliferating cell growth. The term is meant to include cancerous growths, e.g., tumors; oncogenic processes, metastatic tissues, and malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. The cancer cells can form the solid tumors or the excessive tumor cells in blood (e.g., hematologic cancer). Alternatively or additionally it can include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. Examples of solid tumors include malignancies, e.g., sarcomas, adenocarcinomas, and carcinomas, of the various organ systems, such as those affecting liver, lung, breast, lymphoid, gastrointestinal (e.g., colon), genitourinary tract (e.g., renal, urothelial cells), prostate and pharynx. Adenocarcinomas include malignancies such as most colon cancers, rectal cancer, renal-cell carcinoma, liver cancer, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus. Examples of cancers that can be treated by the methods described herein include e.g., bone cancer, pancreatic cancer, skin cancer (e.g., melanoma), cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the

endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin Disease, non-Hodgkin lymphoma, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemias including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, and/or T-cell lymphoma.

As used herein, the term “vector” refers to a vehicle by which a polynucleotide sequence (e.g. a foreign gene) can be introduced into a host cell, in order to obtain the desired gene expression of the introduced nucleotide sequence. Cloning vectors can include e.g., plasmids, phages, viruses, etc. Most popular type of vector is a "plasmid", which refers to a closed circular double stranded DNA loop into which additional DNA segments comprising gene of interest can be ligated. Another type of vector is a viral vector, in which a nucleic acid construct to be transported is ligated into the viral genome. Viral vectors are capable of autonomous replication in a host cell into which they are introduced or may integrate themselves into the genome of a host cell and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In some embodiments, the vectors are viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses).

As used herein, a "subject" is a mammal, such as a human or a non-human animal. In some embodiments, the subject, e.g., patient, to whom the cells, cell populations, or compositions are administered 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 dog, a cat, a horse, a rodent, a rat, or a mouse.

As used herein, the term “about” refers to a measurable value such as an amount, a time

duration, and the like, and encompasses variations of $\pm 20\%$, $\pm 10\%$, $\pm 5\%$, $\pm 1\%$, $\pm 0.5\%$ or $\pm 0.1\%$ from the specified value.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

Exemplary embodiments are illustrated in referenced figures. It is intended that the embodiments and figures disclosed herein are to be considered illustrative rather than restrictive.

FIG. 1A is a schematic diagram showing mouse IL-12 expression vectors. P2A encodes a 2A self-cleaving peptide; mSP encodes a mouse signal peptide; mP40 encodes mouse IL-12B (beta chain); the linker encodes a peptide linker; mP35 encodes mouse IL-12A (alpha chain); the hinge encodes an immunoglobulin hinge region; mCH2 encodes mouse immunoglobulin IgG4 heavy chain constant domain CH2; mCH3 encodes mouse immunoglobulin IgG4 heavy chain constant domain CH3; mCD4 TM encodes a mouse CD4 transmembrane region; NHS-76 vH encodes the heavy chain variable region (VH) of a tumor necrosis-targeting human IgG1 (NHS-76); and NHS-76 vL encodes the light chain variable region (VL) of NHS-76.

FIG. 1B is a schematic diagram showing human IL-12 expression vectors. P2A encodes a 2A self-cleaving peptide; hSP encodes a human signal peptide; hP40 encodes human IL-12B; the linker encodes a peptide linker; hP35 encodes human IL-12A; the hinge encodes an immunoglobulin hinge region; hmCH2 (human mutated CH2) encodes mutated human immunoglobulin IgG4 heavy chain constant domain CH2; hCH3 (human CH3) encodes human immunoglobulin IgG4 heavy chain constant domain CH3; hCD4 TM encodes a human CD4

transmembrane region; NHS-76 vH encodes the VH of NHS-76; and NHS-76 vL encodes the VL of NHS-76.

FIG. 1C is a schematic diagram showing vectors expressing human IL-12 in combination with a TCR. TCR encodes a T-cell receptor (e.g., L202); P2A encodes a 2A self-cleaving peptide;

hSP encodes a human signal peptide; hP40 encodes human IL-12B; the linker encodes a peptide linker; hP35 encodes human IL-12A; the hinge encodes an immunoglobulin hinge region; hmCH2 encodes mutated human immunoglobulin IgG4 heavy chain constant domain CH2; hCH3 encodes human immunoglobulin IgG4 heavy chain constant domain CH3; hCD4 TM encodes a human CD4 transmembrane region; NHS-76 vH encodes the VH of NHS-76; and NHS-76 vL encodes the VL of NHS-76.

FIG. 1D is a schematic diagram showing vectors expressing human IL-12 in combination with a CAR. CAR encodes a chimeric antigen receptor; P2A encodes a 2A self-cleaving peptide; hSP encodes a human signal peptide; hP40 encodes human IL-12B; the linker encodes a peptide linker; hP35 encodes human IL-12A; the hinge encodes an immunoglobulin hinge region; hmCH2 encodes mutated human immunoglobulin IgG4 heavy chain constant domain CH2; hCH3 encodes human immunoglobulin IgG4 heavy chain constant domain CH3; hCD4 TM encodes a human CD4 transmembrane region; NHS-76 vH encodes the VH of NHS-76; and NHS-76 vL encodes the VL of NHS-76.

FIG. 2A is a histogram showing mouse IL-12 concentration in culture media as determined by ELISA (enzyme-linked immunosorbent assay). Purified mouse lymphocytes were untransduced (Sample Name: UT), or transduced to express the L202 TCR alone (Sample Name: L202), L202 plus mouse membrane-tethered IL-12 (Sample Name: mt12), or L202 plus NHS76-IL-12 (Sample Name: NHS76). The lymphocytes were co-cultured with LLC-HLA-A2-Peplinker (LLW) (target cells), or cultured alone (Control).

FIG. 2B is a histogram showing mouse IFN γ concentration in the culture medium as determined by ELISA. Purified mouse lymphocytes were untransduced (Sample Name: UT), or transduced to express the L202 TCR alone (Sample Name: L202), L202 plus mouse membrane-tethered IL-12 (Sample Name: mt12), or L202 plus NHS76-IL-12 (Sample Name: NHS76). The lymphocytes were co-cultured with LLC-HLA-A2-Peplinker (LLW) (target cells), or cultured alone (Control).

FIG. 3 is a histogram showing mouse IFN γ concentration in the culture medium as determined by ELISA. Purified mouse lymphocytes were untransduced (Sample Name: UT), or transduced to express the L202 TCR alone (Sample Name: L202), L202 plus membrane-tethered IL-12 (Sample Name: mt12), or L202 plus NHS76-IL-12 (Sample Name: NHS76). The lymphocytes were co-cultured with Jurkat cells (target cells+mouse lymphocytes), or cultured alone (Control).

FIG. 4A is a graph showing cell surface IL-12 expression in untransduced primary mouse lymphocytes (Sample Name: untransduced).

FIG. 4B is a graph showing cell surface IL-12 expression in primary mouse lymphocytes that were transduced to express L202 TCR (Sample Name: L202).

FIG. 4C is a graph showing cell surface IL-12 expression in primary mouse lymphocytes that were transduced to express L202 TCR and human membrane-tethered IL-12 (Sample Name: L202+mtIL12).

FIG. 4D is a graph showing cell surface IL-12 expression in primary mouse lymphocytes that were transduced to express L202 TCR and NHS76-IL12 (Sample Name: L202+NHS-IL12).

FIG. 5A is a graph showing human Fab (hFab) expression in untransduced primary mouse lymphocytes (Sample Name: NHS76-12 UT) or primary mouse lymphocytes that were transduced to express NHS76-IL-12 (Sample Name: NHS76-12). NHS76-IL12 was detected by staining for human Fab.

FIG. 5B is a graph showing IL-12 expression in untransduced primary mouse lymphocytes (Sample Name: NHS76-12 UT) or primary mouse lymphocytes that were transduced to express NHS76-IL-12 (Sample Name: NHS76-12). NHS76-IL12 exhibited a peak shift when stained for IL-12.

FIG. 6A is a graph showing TCR β surface staining of untransduced human PBMCs (Sample Name: untransduced).

FIG. 6B is a graph showing TCR β surface staining of human PBMCs that were transduced to express L202 TCR in combination with human long membrane-tethered IL-12 (Sample Name: L202+long mtIL12).

FIG. 6C is a graph showing TCR β surface staining of human PBMCs that were transduced to express L202 TCR in combination with human membrane-tethered IL-12 (Sample Name: L202+mtIL12).

FIG. 6D is a graph showing TCRb surface staining of human PBMCs that were transduced to express L202 TCR alone (Sample Name: L202).

FIG. 6E is a graph showing human IL-12 surface staining of untransduced human PBMCs (Sample Name: untransduced).

FIG. 6F is a graph showing human IL-12 surface staining of human PBMCs that were transduced to express L202 TCR in combination with long mt-IL-12 (Sample Name: L202+long mtIL12).

FIG. 6G is a graph showing human IL-12 surface staining of human PBMCs that were transduced to express L202 TCR in combination with mt-IL-12 (Sample Name: L202+mtIL12).

FIG. 6H is a graph showing human IL-12 surface staining of human PBMCs that were transduced to express L202 TCR alone (Sample Name: L202).

FIG. 7A is a graph showing CD69 expression in human PBMCs co-cultured with A375-HLA-A2-peplinker (LLW) melanoma target cells. Samples included untransduced human PBMCs (Sample Name: UT A375LLW); human PBMCs transduced to express L202 TCR (Sample Name: L202 A375LLW); human PBMCs transduced to express L202 plus human membrane-tethered IL-12 (Sample Name: smt A375LLW); and human PBMCs transduced to express L202 plus human long membrane-tethered IL-12 (lmt) (Sample Name: lmt A375LLW).

FIG. 7B is a graph showing CD69 expression in human PBMCs without co-culturing. Samples included untransduced human PBMCs (Sample Name: UT); human PBMCs transduced to express L202 TCR (Sample Name: L202); human PBMCs transduced to express L202 plus human membrane-tethered IL-12 (Sample Name: smt); and human PBMCs transduced to express L202 plus human long membrane-tethered IL-12 (Sample Name: lmt).

FIG. 8A shows a flow cytometry result showing human IFN γ (hIFN γ) expression in untransduced human PBMCs.

FIG. 8B shows a flow cytometry result showing human IFN γ (hIFN γ) expression in human PBMCs that were transduced to express L202 TCR.

FIG. 8C shows a flow cytometry result showing human IFN γ (hIFN γ) expression in human PBMCs that were transduced to express L202 TCR plus membrane-tethered IL-12.

FIG. 8D shows a flow cytometry result showing human IFN γ (hIFN γ) expression in human PBMCs that were transduced to express L202 TCR plus long membrane-tethered IL-12.

FIG. 8E shows a flow cytometry result showing human IFN γ (hIFN γ) expression in untransduced human PBMCs. The PBMCs were co-cultured with A375-HLA-A2-peplinker (LLW) melanoma target cells.

FIG. 8F shows a flow cytometry result showing human IFN γ (hIFN γ) expression in human PBMCs that were transduced to express L202 TCR. The PBMCs were co-cultured with A375-HLA-A2-peplinker (LLW) melanoma target cells.

FIG. 8G shows a flow cytometry result showing human IFN γ (hIFN γ) expression in human PBMCs that were transduced to express L202 TCR plus membrane-tethered IL-12. The PBMCs were co-cultured with A375-HLA-A2-peplinker (LLW) melanoma target cells.

FIG. 8H shows a flow cytometry result showing human IFN γ (hIFN γ) expression in human PBMCs that were transduced to express L202 TCR plus long membrane-tethered IL-12. The PBMCs were co-cultured with A375-HLA-A2-peplinker (LLW) melanoma target cells.

FIG. 9A is a graph showing CD69 expression in the mTCRb $^{+}$ population after creating a mixed population of 50% L202 TCR positive (mTCRb $^{+}$) PBMCs (including untransduced PBMCs as a control) and 50% untransduced human PBMCs (mTCRb $^{-}$). Samples included untransduced PBMCs (Sample Name: UT+A375); PBMCs transduced to express L202 TCR (Sample Name: L202+A375); PBMCs transduced to express L202 plus short membrane-tethered IL-12 (smt) (Sample Name: smt+ A375); and PBMCs transduced to express L202 plus long membrane-tethered IL-12 (lmt) (Sample Name: lmt+A375).

FIG. 9B is a graph showing CD69 expression in mTCRb $^{-}$ population after creating a mixed population of 50% L202 TCR positive (mTCRb $^{+}$) PBMCs (including untransduced PBMCs as a control) and 50% untransduced PBMCs (mTCRb $^{-}$). Samples included untransduced PBMCs (Sample Name: UT+A375); PBMCs transduced to express L202 TCR (Sample Name: L202+A375); PBMCs transduced to express L202 plus short membrane-tethered IL-12 (smt) (Sample Name: smt+ A375); and PBMCs transduced to express L202 plus long membrane-tethered IL-12 (lmt) (Sample Name: lmt+A375).

FIG. 10A shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb $^{+}$ cells from untransduced PBMCs

FIG. 10B shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb $^{+}$ cells from PBMCs that were transduced to express L202 TCR.

FIG. 10C shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁺ cells from PBMCs that were transduced to express L202 TCR plus human membrane-tethered IL-12.

FIG. 10D shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁺ cells from PBMCs that were transduced to express L202 TCR plus human long membrane-tethered IL-12.

FIG. 10E shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁺ cells from untransduced PBMCs. The PBMCs were co-cultured with A375-LLW target cell.

FIG. 10F shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁺ cells from PBMCs that were transduced to express L202 TCR. The PBMCs were co-cultured with A375-LLW target cell.

FIG. 10G shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁺ cells from PBMCs that were transduced to express L202 TCR plus human membrane-tethered IL-12. The PBMCs were co-cultured with A375-LLW target cell.

FIG. 10H shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁺ cells from PBMCs that were transduced to express L202 TCR plus human long membrane-tethered IL-12. The PBMCs were co-cultured with A375-LLW target cell.

FIG. 11A shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁻ cells from untransduced PBMCs

FIG. 11B shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁻ cells from PBMCs that were transduced to express L202 TCR.

FIG. 11C shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁻ cells from PBMCs that were transduced to express L202 TCR plus human membrane-tethered IL-12.

FIG. 11D shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁻ cells from PBMCs that were transduced to express L202 TCR plus human long membrane-tethered IL-12.

FIG. 11E shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb⁻ cells from untransduced PBMCs. The PBMCs were co-cultured with A375-LLW target cell.

FIG. 11F shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb- cells from PBMCs that were transduced to express L202 TCR. The PBMCs were co-cultured with A375-LLW target cell.

FIG. 11G shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb- cells from PBMCs that were transduced to express L202 TCR plus human membrane-tethered IL-12. The PBMCs were co-cultured with A375-LLW target cell.

FIG. 11H shows a flow cytometry result showing human IFN γ (hIFN γ) expression in mTCRb- cells from PBMCs that were transduced to express L202 TCR plus human long membrane-tethered IL-12. The PBMCs were co-cultured with A375-LLW target cell.

FIG. 12A shows a flow cytometry result showing CD4 and CD8 expression in untransduced PBMCs (UT).

FIG. 12B shows a flow cytometry result showing CD4 and CD8 expression in L202 TCR-T PBMCs (L202).

FIG. 12C shows a flow cytometry result showing CD4 and CD8 expression in L202 TCR-T PBMCs expressing human membrane-tethered IL-12 (mt-IL12).

FIG. 12D shows a flow cytometry result showing CD4 and CD8 expression in L202 TCR-T PBMCs expressing human long membrane-tethered IL-12 (lmt-IL12).

FIG. 13 shows a flow cytometry result showing percentages of effector memory CD4⁺ cells and central memory CD4⁺ cells in untransduced PBMCs.

FIG. 14A shows a flow cytometry result showing percentages of effector memory CD4⁺ cells and central memory CD4⁺ cells in untransduced PBMCs (UT).

FIG. 14B shows a flow cytometry result showing percentages of effector memory CD4⁺ cells and central memory CD4⁺ cells in L202 TCR-T PBMCs (L202).

FIG. 14C shows a flow cytometry result showing percentages of effector memory CD4⁺ cells and central memory CD4⁺ cells in L202 TCR-T PBMCs expressing human membrane-tethered IL-12 (mt-IL12).

FIG. 14D shows a flow cytometry result showing percentages of effector memory CD4⁺ cells and central memory CD4⁺ cells in L202 TCR-T PBMCs expressing human long membrane-tethered IL-12 (lmt-IL12).

FIG. 15A is an image of flow cytometry results showing percentages of effector memory CD8⁺ cells and central memory CD4⁺ cells in untransduced PBMCs (UT).

FIG. 15B is an image of flow cytometry results showing percentages of effector memory CD8+ cells and central memory CD4+ cells in L202 TCR-T PBMCs (L202).

FIG. 15C is an image of flow cytometry results showing percentages of effector memory CD8+ cells and central memory CD4+ cells in L202 TCR-T PBMCs expressing human membrane-tethered IL-12 (mt-IL12).

FIG. 15D is an image of flow cytometry results showing percentages of effector memory CD8+ cells and central memory CD4+ cells in L202 TCR-T PBMCs expressing human long membrane-tethered IL-12 (lmt-IL12).

FIG. 16 shows IL-12 concentrations in cell culture medium of untransduced human PBMCs (Sample Name: UT), human PBMCs transduced to express human IL-12 (Sample Name: IL-12), human PBMCs transduced to express human NHS76-IL-12 (Sample Name: NHS76-IL-12), or human PBMCs transduced to express human membrane-tethered IL-12 (Sample Name: Mt-IL-12).

FIG. 17 lists sequences that are described in the present disclosure.

DETAILED DESCRIPTION

The human immune system is capable of recognizing and eliminating cells that have become infected or damaged as well as those that have become cancerous. Immune cell therapy takes advantage of the human immune system and is revolutionizing cancer therapy. It involves the transfer of immune cells into a patient. The cells are most commonly derived from the immune system and can originate from the patient or from another individual. In autologous cancer immunotherapy, immune cells are extracted from the patient, genetically modified and cultured *in vitro*, and returned to the same patient. Comparatively, allogeneic therapies involve cells isolated and expanded from a donor subject.

Many different kinds of immune cells are used in immune cell therapies. These cell therapies include e.g., tumor-infiltrating lymphocyte (TIL) therapy, engineered T cell receptor (TCR) therapy, chimeric antigen receptor (CAR) T cell therapy, and natural killer (NK) cell therapy.

Cancer patients have naturally occurring T cells that are often capable of targeting their cancer cells. Tumor-infiltrating lymphocyte (TIL) therapy involves harvesting these naturally occurring T cells from the patient, then activating and expanding them. These activated T cells

are then re-infused into the patient. In contrast, engineered T cell receptor (TCR) therapy involves isolating T cells from patients, but instead of just activating and expanding the available anti-tumor T cells, the T cells are also transfected with a vector encoding a new T cell receptor that allows T cells to target specific cancer antigens presented by major histocompatibility complex (MHC). Chimeric antigen receptor (CAR) T cell therapy is similar to TCR therapy. In CAR T cell therapy, the cells are transfected by a vector encoding a chimeric antigen receptor. The chimeric antigen receptor can bind to cancer antigens and does not require that the cancer antigens be presented by MHC. Some other immune cells can also be used in these cell therapies. For example, natural killer cells can also be transfected with a vector encoding a chimeric antigen receptor.

The present disclosure shows that immune cell therapies can be synergistically combined with Interleukin-12 (IL-12). However, IL-12 administration is commonly associated with severe toxicity, including e.g., hematologic toxicities, anemia, lymphopenia, neutropenia, muscle and hepatic toxicities, and even in some cases, death. The toxicities of IL-12 administration greatly limits its use in immune cell therapies.

The present disclosure provides an improved immune cell therapy, wherein the engineered cells (e.g., immune cells, T cells, NK cells, tumor infiltrating cells) produce IL-12 in a controlled fashion and deliver IL-12 directly to the target site, thereby significantly improving efficacy and safety. In one aspect, the IL-12 is a membrane-tethered IL-12, which further limits the effects of IL-12 to the local target area. In another aspect, the IL-12 is conjugated to scFv of NHS76, which is an antibody that targets a tumor's necrosis center.

The expression of IL-12 further improves the efficacy of immune cell therapies. Antigen escape and downregulation (e.g., antigen loss) have emerged as major issues impacting the durability of immune cell therapy. Particularly, in solid tumors, most antigens are expressed at lower levels and/or more heterogeneously. Because of the heterogeneity, certain tumor cells can escape immune cell therapy targeting a specific antigen by decreasing the expression of that antigen. The immune pressure by the immune cell therapy can drive cancer cells to evolve by modulating expression of their target antigens, through either loss of detectable antigen or diminished expression of the antigen to a level below a threshold required for immune cell activity. Once the majority of the tumor cells are killed, those remaining tumor cells that are resistant to the immune cell therapy quickly grow and the entire recurrent solid tumor becomes

resistant the immune cell therapy. Antigen loss or antigen-low escape has become a greater barrier for treating solid tumors. Details of antigen loss mechanisms and relevant clinical data can be found, e.g., in Majzner and Crystal, "Tumor antigen escape from CAR T-cell therapy." *Cancer discovery* 8.10 (2018): 1219-1226, which is incorporated herein by reference by its entirety. The expression of IL-12 in engineered immune cells can further increase immune response (e.g., stimulating surrounding immune cells) at the local tumor microenvironment, thereby killing surrounding cancer cells that do not express those antigens or express a low level of antigens, preventing any cancer cells from escaping the immune cell therapy.

In one aspect, the present disclosure relates to IL-12 armored immune cell therapies (e.g., TCR-T, CAR-T, CAR-NK or TIL). In some embodiments, the IL-12 is modified by fusing to one or more (e.g., 1, 2, 3, 4, or 5) immunoglobulin constant domains and/or a membrane-tethering region (e.g., a transmembrane region), such that the IL-12 is tethered to the cell membrane. In some embodiments, the IL-12 is modified by linking it to variable regions (e.g., VH and VL) of a tumor-targeting antibody. These IL-12 armored immune cell therapies can boost anti-tumor immunity or efficacy, induce host immune system activation, induce epitope spreading, and/or increase safety in humans. The disclosure also provides vectors, cells comprising such vectors, and methods of making such vectors. Also provided are methods of using the IL-12 armored immune cell therapies of the disclosure, including but not limited to, treating various cancers and some other disorders in a human.

IL-12 AND IMMUNE CELL THERAPY

IL-12 is a heterodimeric molecule composed of an alpha chain (the p35 subunit) and a beta chain (the p40 subunit) covalently linked by a disulfide bridge to form the biologically active 70 kDa dimer. Biologically, IL-12 is an inflammatory cytokine that is produced in response to infection by a variety of cells of the immune system, including phagocytic cells, B cells and activated dendritic cells. IL-12 plays an essential role in mediating the interaction of the innate and adaptive arms of the immune system, acting on T-cells and natural killer (NK) cells to enhance the proliferation and activity of cytotoxic lymphocytes and the production of other inflammatory cytokines, especially interferon-gamma (IFN-gamma). Detailed description of IL-12 can be found in, e.g., Colombo *et al.*, "Interleukin-12 in anti-tumor immunity and immunotherapy." *Cytokine & growth factor reviews* 13.2 (2002): 155-168; and Hamza *et al.*,

“Interleukin 12 a key immunoregulatory cytokine in infection applications.” International journal of molecular sciences 11.3 (2010): 789-806; which are incorporated herein by reference in their entirety.

The IL-12 receptor is expressed in a constitutive or inducible manner in a variety of immune cells, including NK cells and T and B lymphocytes. Ligand-bound IL-12R becomes phosphorylated on tyrosines, which provides harboring sites for two kinases, JAK2 and TYK2. Among the STAT family of transcription factors, STAT4 is considered to be the most specific mediator of cellular responses elicited by IL-12. A remarkable function of IL-12 is its ability to induce IFN γ release from natural killer (NK) cells as well as CD4⁺ and CD8⁺ T cells. In fact, IL-12 signaling via STAT-4 is critical for Th1 differentiation and the acquisition of cytolytic functions by CD8⁺ T cells. IFN γ in turn strongly modifies the tumor microenvironment. The best studied beneficial mechanisms of IFN γ are: 1) enhancing MHC I antigen presentation by tumor cells; 2) inducing the expression of CXCL9, 10, and 11 chemokines to attract NK, Th1, and CD8⁺ T cells; 3) transforming M2 macrophages into activated antitumor M1 macrophages; and 4) acting on endothelial cells to mediate anti-angiogenesis in a CXCR3-dependent fashion while also enhancing the expression of homing receptors for T-cell recruitment.

Although the potent antitumor effects of IL-12 are well established, IL-12 administration also elicit severe side effects. These side effects associated with systemic administration of IL-12 in clinical investigations and the very narrow therapeutic index of this cytokine have markedly limited the use of IL-12 as anti-cancer therapy. A detailed description of IL-12 can be found, e.g., in Lasek *et al.*, "Interleukin 12: still a promising candidate for tumor immunotherapy?." Cancer Immunology, Immunotherapy 63.5 (2014): 419-435; Berraondo *et al.*, "Revisiting interleukin-12 as a cancer immunotherapy agent." Clinical Cancer Research 24.12 (2018): 2716-2718; which are incorporated herein by reference in their entirety.

In order to improve efficacy and reduce toxicity, the present disclosure provides a modified IL-12 and/or an improved way to deliver IL-12. Particularly, IL-12 is expressed in immune cells that target cancer cells. These engineered cells (e.g., immune cells, T cells, NK cells, tumor-infiltrating cells) can produce IL-12 in a controlled fashion and deliver it directly to the target site. In one aspect, the IL-12 is a membrane-tethered IL-12, which further limits the effects of IL-12 to the local target area.

In some embodiments, the modified IL-12 proteins can be any of the fusion proteins described herein. In some embodiments, provided herein is a fusion protein comprising an IL-12 alpha subunit and an IL-12 beta subunit. The IL-12 proteins can be derived from any species. In some embodiments, the IL-12 alpha subunit is a human IL-12 alpha subunit. In some embodiments, the IL-12 alpha subunit is a mouse IL-12 alpha subunit. In some embodiments, the IL-12 beta subunit is a human IL-12 beta subunit. In some embodiments, the IL-12 beta subunit is a mouse IL-12 beta subunit. In some embodiments, the IL-12 alpha subunit and beta subunit are connected by a polypeptide linker sequence.

In some embodiments, the amino acid sequence of the IL-12 subunits (e.g., alpha or beta) can be modified. In some embodiments, the IL-12 subunits, includes one or more amino acid variations, e.g., substitutions, deletions, insertions, and/or mutations, compared to the sequence of a wild-type molecule, e.g., any IL-12 subunits described herein. Exemplary variants include those designed to improve the binding affinity to IL-12 receptors and/or other biological properties of the IL-12 subunits (e.g., protein stability). Amino acid sequence variants of an IL-12 subunit can be prepared by introducing appropriate modifications into the nucleotide sequence encoding the IL-12 alpha and/or beta subunits, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into, and/or substitutions of residues within the amino acid sequences of the IL-12 alpha and/or beta subunits. Any combination of such deletions, insertions, and substitutions can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., specifically binding to the IL-12 receptor.

In some embodiments, the IL-12 alpha subunit has an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 15. In some embodiments, the IL-12 beta subunit has an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 16. In some embodiments, the IL-12 alpha and beta subunits are connected by a linker peptide sequence GGGGSGGGGSGGGGS (SEQ ID NO: 17). In some embodiments, the IL-12 fusion protein described herein has an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 8.

In some embodiments, the fusion protein described herein comprises a membrane-tethering region. In some embodiments, the membrane-tethering region is a transmembrane region of a transmembrane protein (e.g., CD4). In some embodiments, the transmembrane region is a transmembrane domain of 4-1BB/CD137, an alpha chain of a T cell receptor, a beta chain of a T cell receptor, B7 (e.g., B7-1), CD3 epsilon, CD4, CD5, CD8, CD8 alpha, CD9, CD16, CD19, CD22, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD134, CD137, CD154, or a zeta chain of a T cell receptor, or any combination thereof. In some embodiments, the fusion protein comprise a transmembrane domain of a membrane immunoglobulin (mIg). In some embodiments, the fusion protein comprises the transmembrane domain of CD28, CD3-zeta, CD3-alpha, or CD3-beta transmembrane. In some embodiments, the fusion protein described herein comprises 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more constant domains of immunoglobulins. In some embodiments, the constant domains are heavy chain constant domains. In some embodiments, the constant domains are light chain constant domains. In some embodiments, the protein comprises 2 constant domains. In some embodiments, the 2 constant domains are CH2 and CH3 domains. In some embodiments, the 2 constant domains are CH1 and CH3 domains. In some embodiments, the 2 constant domains are CH1 and CH2 domains. In some embodiments, the 2 constant domains are both CH1 domains. In some embodiments, the 2 constant domains are both CH2 domains. In some embodiments, the 2 constant domains are both CH3 domains. In some embodiments, the protein comprises the entire Fc region of the immunoglobulin. In some embodiments, the protein comprises 3 constant domains. In some embodiments, the 3 constant domains are two CH2 and one CH3. In some embodiments, the 3 constant domains are linked sequentially as CH2-CH2-CH3. In some embodiments, the 3 constant domains are linked sequentially as CH1-CH2-CH3. In some embodiments, the protein comprises 1, 2, 3, 4, 5 or more CH2 domains. In some embodiments, the protein comprises 1, 2, 3, 4, 5 or more CH3 domains. In some embodiments, the immunoglobulin is a human immunoglobulin. In some embodiments, the immunoglobulin is a mouse immunoglobulin. In some embodiments, the immunoglobulin is an immunoglobulin G (IgG), an IgM, an IgE, an IgA, or an IgD molecule. In some embodiments, the immunoglobulin is an IgG1, IgG2, IgG3, or IgG4. In some embodiments, the immunoglobulin is a human IgG4. In some embodiments, the constant domains are from the same immunoglobulin class (e.g., IgG, IgM, IgA). In some embodiments, the constant domains comprise CH2, CH3, and CH4 of IgM.

In some embodiments, the constant domain is a wild-type constant domain from human. In some embodiments, the constant domain is a mutated human constant domain. In some embodiments, the constant domain is a mutated human immunoglobulin IgG4 heavy chain constant domain. In some embodiments, the constant domain is a wild-type human immunoglobulin IgG4 heavy chain constant domain CH2. In some embodiments, the constant domain is a mutated human immunoglobulin IgG4 heavy chain constant domain CH2. In some embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acids are mutated in the CH2 domain. In some embodiments, at least or about 1%, 2%, 3%, 4%, 5%, 6% or 7% of all amino acid residues of the CH2 domain are mutated. In some embodiments, the constant domain is a wild-type human immunoglobulin IgG4 heavy chain constant domain CH3. In some embodiments, the constant domain is a mutated human immunoglobulin IgG4 heavy chain constant domain CH3. In some embodiments, the mutated constant domain described herein (e.g., hmCH2) has a reduced binding affinity to soluble FcγR as compared to a corresponding wild-type constant domain. In some embodiments, the mutated constant domain described herein (e.g., hmCH2) has an enhanced T cell persistence *in vivo* as compared to a corresponding wild-type constant domain. In some embodiments, the mutations in the CH2 domain are L235E and/or N297Q according to EU numbering. In some embodiments, the mutation includes one or more of the following: N297A, N297Q or N297G (EU numbering). In some embodiments, the mutation is L234A/L235A (LALA) (EU numbering). In some embodiments, the mutation is F234A and/or L235A. In some embodiments, the mutation includes one or more of the following: H268Q, V309L, A330S and/or P331S (EU numbering). In some embodiments, the mutation includes one or more of the following: V234A, G237A, P238S, H268A, V309L, A330S, and/or P331S. Detailed descriptions can be found, e.g., in Jonnalagadda *et al.*, "Chimeric antigen receptors with mutated IgG4 Fc spacer avoid fc receptor binding and improve T cell persistence and antitumor efficacy." *Molecular Therapy* 23.4 (2015): 757-768; and U.S. Patent No. 9,914,909 B2 each of which is incorporated herein by reference in its entirety.

In some embodiments, the CH2 domain described herein comprises the amino acid sequence set forth in any of SEQ ID NO: 41, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the CH2 domain described herein is encoded by a nucleic acid sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to

SEQ ID NO: 42. In some embodiments, the CH3 domain described herein comprises the amino acid sequence set forth in any of SEQ ID NO: 43, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the CH3 domain described herein is encoded by a nucleic acid sequence that is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 44.

In some embodiments, the constant domains are linked to the IL-12 subunits with a hinge region. In some embodiments, the amino acid sequence of the constant domain described herein can be modified. In some embodiments, the constant domain (e.g., CH2 or CH3), include one or more amino acid variations, e.g., substitutions, deletions, insertions, and/or mutations, compared to the sequence of a wild-type molecule, e.g., any constant domains described herein.

In some embodiments, the fusion protein is a membrane-tethered protein. In some embodiments, the membrane-tethering region is fused to the constant domains as described herein. In some embodiments, the membrane-tethered IL-12 fusion protein described herein has an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 10. In some embodiments, the membrane-tethered IL-12 fusion protein described herein has an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 12.

In some embodiments, the fusion protein is a soluble protein. In some embodiments, the fusion protein comprises a signal peptide (e.g., a human signal polypeptide). In some embodiments, the signal peptide sequence is a secretion signal peptide.

Another strategy for improving the safety of IL-12, is to direct their delivery to tumors via fusion to a tumor-targeting antibody. Such antibody-cytokine fusion proteins, or “immunocytokines,” have previously demonstrated the ability to enhance anti-tumor immunity in preclinical models. To maximize immunocytokine tolerability, the antibody selected as a vehicle usually binds specifically to a tumor-associated antigen. For example, antibodies directed against necrosis-associated antigens, which are abundantly present in tumors but not in normal tissues, offer an attractive delivery approach. In one aspect, these antibody-cytokine fusion proteins are expressed in tumor-targeting cells (e.g., immune cells, T cells, NK cells, tumor-

infiltrating cells); thus, these antibody-cytokine fusion proteins are expressed specifically at the target site, which further improves their safety.

In some embodiments, the fusion protein comprises a tumor-targeting antibody or antigen-binding fragment thereof. The tumor-targeting antibody or antigen binding fragment can deliver IL-12 to the target site, thereby further reducing the side effects of IL-12. In some embodiments, the fusion protein is not a membrane-tethered protein and/or does not have a transmembrane protein. The tumor-targeting antibody or antigen-binding fragment can target a tumor-associated antigen. As used herein, the term “tumor associated antigen” refers to an antigen that is or can be presented on a tumor cell surface and that is located on or within tumor cells. In some other embodiments, the tumor associated antigens can be exclusively expressed on tumor cells or may represent a tumor specific mutation compared to non-tumor cells. In some other embodiments, the tumor associated antigens can be found in both tumor cells and non-tumor cells, but is overexpressed on tumor cells when compared with non-tumor cells, or is more accessible for antibody binding in tumor cells due to the less compact structure of the tumor tissue compared to non-tumor tissue. In some embodiments the tumor associated antigen is located on the vasculature of a tumor. Illustrative examples of tumor associated surface antigens include CD10, CD19, CD20, CD22, CD21, CD22, CD25, CD30, CD33, CD34, CD37, CD44v6, CD45, CD133, Fms-like tyrosine kinase 3 (FLT-3, CD135), chondroitin sulfate proteoglycan 4 (CSPG4, melanoma-associated chondroitin sulfate proteoglycan), Epidermal growth factor receptor (EGFR), Her2neu, Her3, IGFR, IL3R, fibroblast activating protein (FAP), CDCP1, Derlin1, Tenascin, frizzled 1-10, the vascular antigens VEGFR2 (KDR/FLK1), VEGFR3 (FLT4, CD309), PDGFR-alpha (CD140a), PDGFR-beta (CD140b), Endoglin, CLEC14, Tem1-8, and Tie2, A33, CAMPATH-1 (CDw52), Carcinoembryonic antigen (CEA), Carboanhydrase IX (MN/CA IX), de2-7 EGFR, EGFRvIII, EpCAM, Ep-CAM, Folate-binding protein, G250, Fms-like tyrosine kinase 3 (FLT-3, CD135), c-Kit (CD117), CSF1R (CD115), HLA-DR, IGFR, IL-2 receptor, IL3R, MCSP (Melanoma-associated cell surface chondroitin sulfate proteoglycane), Muc-1, Prostate-specific membrane antigen (PSMA), Prostate stem cell antigen (PSCA), Prostate specific antigen (PSA), and TAG-72.

In some embodiments, provided herein is a fusion protein comprising an IL-12 alpha subunit and an IL-12 beta subunit. In some embodiments, the protein further comprises a heavy chain variable region and a light chain variable region of a tumor-targeting antibody or antigen

binding fragment thereof. In some embodiments, the tumor-targeting antibody or antigen binding fragment thereof is a scFv. In some embodiments, the tumor necrosis-targeting antibody is a human antibody. In some embodiments, the tumor necrosis-targeting antibody is a human IgG1. In some embodiments, the tumor necrosis-targeting antibody is NHS76. NHS76 is a fully human, phage display-derived IgG1 antibody selected for its specific ability to bind to necrotic regions and thereby target to tumors *in vivo*. Detailed descriptions can be found, e.g., in Fallon *et al.*, "The immunocytokine NHS-IL12 as a potential cancer therapeutic." *Oncotarget* 5.7 (2014): 1869; and Sharifi *et al.*, "Characterization of a phage display-derived human monoclonal antibody (NHS76) counterpart to chimeric TNT-1 directed against necrotic regions of solid tumors." *Hybridoma and hybridomics* 20.5-6 (2001): 305-312; each of which is incorporated herein by reference in its entirety. Thus, in some embodiments, the antibody-cytokine fusion proteins has an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 14.

T CELL RECEPTORS AND BINDING MOLECULES

T cells are a type of lymphocyte which typically develops in the thymus gland and plays a central role in the adaptive immune response. T cells are distinguished from other lymphocytes by the presence of a T-cell receptor on the cell surface. Differentiated T cells have an important role in controlling the immune response. CD8⁺ T cells, also known as "killer cells", are cytotoxic. Once they recognize a target cell, they are able to directly kill the target cell (e.g., virus-infected cells or cancer cells). CD8⁺ T cells also produce cytokines and recruit other cells (e.g., macrophages and natural killer (NK) cells) to mount an immune response. CD4⁺ T cells, also known as "helper cells", can indirectly kill target cells, e.g., by facilitating maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules, which are expressed on the surface of antigen-presenting cells (APCs). Once activated, they divide rapidly and secrete cytokines that regulate or assist the immune response. Regulatory T cells are important for tolerance, thereby preventing or inhibiting autoimmune response. The major role of regulatory T cells is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress autoreactive T cells that escaped the process of negative selection in the thymus.

T cells play an important role in cancer immunity where antigens from the cancer cells are taken up and presented on the cell surface of special immune cells called antigen-presenting cells (APCs) so that other immune cells can recognize the antigens of interest. In the lymph nodes, the APCs activate the T-cells and activate them to recognize the tumor cells. The activated T-cells can then travel via the blood vessels to reach the tumor, infiltrate it, recognize the cancer cells and kill them.

The activation of T cells requires T cell receptors. A “T cell receptor” or “TCR” is a molecule that contains a variable α (or alpha) and β (or beta) chains (also known as TCR α and TCR β , respectively) or a variable γ (or gamma) and δ (or delta) chains (also known as TCR γ and TCR δ , respectively), or antigen-binding portions thereof, and which is capable of specifically binding to an antigen, e.g., a peptide antigen or peptide epitope bound to a major histocompatibility complex (MHC) molecule.

The present disclosure provides a T cell receptor (TCR) or antigen-binding fragment thereof, and binding molecules derived from TCR. In some embodiments, the TCR is in the ab form. 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. Generally, a TCR is found on the surface of T cells (or T lymphocytes) where it is generally responsible for recognizing antigens, such as peptides bound to major histocompatibility complex (MHC) molecules.

In some embodiments, the TCR is an intact or full-length TCR, such as a TCR containing the α chain and β chain. In some embodiments, the TCR is an antigen-binding portion that is less than a full-length TCR but that binds to a specific peptide bound in an MHC molecule, such as binds to an MHC-peptide complex. In some cases, an antigen-binding portion or fragment of a TCR can contain only a portion of the structural domains of a full-length or intact TCR, but yet is able to bind the peptide epitope, such as 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 α (Va or V α) chain and variable β (Vb or V β) chain of a TCR, or antigen-binding fragments thereof sufficient to form a binding site for binding to a specific MHC-peptide complex.

The variable domains of the TCR contain complementarity determining regions (CDRs), which generally are the primary contributors to antigen recognition and binding capabilities and specificity of the peptide, MHC and/or MHC-peptide complex. In some embodiments, a CDR of

a TCR or combination thereof forms all or substantially all of the antigen-binding site of a given TCR molecule. The various CDRs within a variable region of a TCR chain generally are separated by framework regions (FRs), which generally display less variability among TCR molecules as compared to the CDRs. In some embodiments, CDR3 is the main CDR responsible for antigen binding or specificity, or is the most important among the three CDRs on a given TCR variable region for antigen recognition, and/or for interaction with the processed peptide portion of the peptide-MHC complex. In some contexts, the CDR1 of the alpha chain can interact with the N-terminal part of certain antigenic peptides. In some cases, CDR1 of the beta chain can interact with the C-terminal part of the peptide. In some contexts, CDR2 contributes most strongly to or is the primary CDR responsible for the interaction with or recognition of the MHC portion of the MHC-peptide complex.

The a-chain and/or b-chain of a TCR also can contain a constant domain, a transmembrane region and/or a short cytoplasmic tail. In some aspects, each chain (e.g. alpha or beta) 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, for example via the cytoplasmic tail, is associated with invariant proteins of the CD3 complex involved in mediating signal transduction. In some cases, the structure allows the TCR to associate with other molecules like CD3 and subunits thereof. For example, a TCR containing constant domains with a transmembrane region may anchor the protein in the cell membrane and associate with invariant subunits of the CD3 signaling apparatus or complex. The intracellular tails of CD3 signaling subunits (e.g. CD3 γ , CD3 δ , CD3 ϵ and CD3 ζ chains) contain one or more immunoreceptor tyrosine-based activation motif or ITAM and generally are involved in the signaling capacity of the TCR complex.

The exact locus of a domain or region can vary depending on the particular structural or homology modeling or other features used to describe a particular domain. It is understood that reference to amino acids, including to a specific sequence set forth as a SEQ ID NO used to describe domain organization of a TCR are for illustrative purposes and are not meant to limit the scope of the embodiments provided. In some cases, the specific domain (e.g. variable or constant) can be several amino acids (such as one, two, three or four) longer or shorter. In some aspects, residues of a TCR are known or can be identified according to the International Immunogenetics Information System (IMGT) numbering system (see e.g. www.imgt.org;

Lefranc *et al.*, "IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains." *Developmental & Comparative Immunology* 27.1 (2003): 55-77.). The structures and variations of TCR are known in the art, and are described, e.g., in WO 2019/195486, which is incorporated herein by reference in its entirety.

In some embodiments, the a chain and b chain of a TCR each further contain a constant domain. In some embodiments, the a chain constant domain (Ca) and b chain constant domain (Cb) individually are mammalian, such as is a human or a non-human constant domain (e.g., a mouse constant domain). In some embodiments, the constant domain is 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, which variable domains each contain CDRs.

In some aspects, TCRs as described herein can contain a human constant region, such as an alpha chain containing a human Ca region and a beta chain containing a human Cb region. In some embodiments, the TCRs are fully human. In some embodiments, the expression and/or activity of TCRs, such as when expressed in human cells, e.g. human T cells, such as primary human T cells, are not impacted by or are not substantially impacted by the presence of an endogenous human TCR.

In some embodiments, the engineered TCRs are expressed at similar or improved levels on the cell surface, exhibit the similar or greater functional activity (e.g. cytolytic activity) and/or exhibit similar or greater anti-tumor activity, when expressed by human cells that contain or express an endogenous human TCR, such as human T cells, as compared to the level of expression, function activity and/or anti-tumor activity of the same TCR in similar human cells but in which expression of the endogenous TCR has been reduced or eliminated. In some examples an engineered TCR as described herein, when expressed in human T cells, is expressed on the cell surface at a level that is at least or at least about 80%, 85%, 90%, 95%, 100%, 105%, 110%, 115% or 120% of the level of expression of the same TCR when expressed in similar human T cells but in which expression of the endogenous TCR has been reduced or eliminated.

In some embodiments, each of the Ca and Cb domains is human. In some embodiments, the Ca is encoded by the TRAC gene (IMGT nomenclature) or is a variant thereof. In some embodiments, the variant of a Ca contains replacement of at least one non-native cysteine.

In some embodiments, the TCR can be a heterodimer of two chains a and b that are linked,

such as by a disulfide bond or disulfide bonds. In some embodiments, the constant domain of the TCR can contain short connecting sequences in which a cysteine residue forms a disulfide bond, thereby linking the two chains of the TCR. In some embodiments, a TCR can have an additional cysteine residue in each of the a and b chains, such that the TCR contains two disulfide bonds in the constant domains. In some embodiments, each of the constant and variable domains contains disulfide bonds formed by cysteine residues.

In some embodiments, the TCR comprises CDRs, Va and/or Vb and constant region sequences as described herein.

In some embodiments, the TCR is a dimeric TCR (dTTCR). In some embodiments a dTTCR contains a first polypeptide wherein a sequence corresponding to a provided TCR a chain variable region sequence is fused to the N terminus of a sequence corresponding to a TCR a chain constant region extracellular sequence, and a second polypeptide wherein a sequence corresponding to a provided TCR b chain variable region sequence is fused to the N terminus a sequence corresponding to a TCR b chain constant region extracellular sequence, the first and second polypeptides being linked by a disulfide bond.

In some embodiments, a TCR can be cell-bound or in soluble form. In some embodiments, the TCR is in cell-bound form expressed on the surface of a cell.

In some embodiments, the TCR is a single chain TCR (scTCR). The scTCR is a single amino acid strand containing an a chain and a b chain that is able to bind to MHC-peptide complexes. Typically, a scTCR can be generated using methods known to those of skill in the art. These methods are described e.g., in WO 96/13593, WO 96/18105, W099/18129, WO 04/033685, W02006/037960, WO2011/044186; WO 2019 /195486; U.S. Patent No. 7,569,664; each of which is incorporated herein by reference in its entirety.

The TCR, antigen binding fragments thereof, and TCR-derived binding molecules can bind or recognize a peptide epitope associated with an antigen of interest (e.g., a tumor-associated antigen). In some embodiments, the antigen can be a peptide epitope expressed on the surface of a cancer cell and/or a cell infected with a virus. In some embodiments, the antigen is presented in the context of an MHC molecule. Such binding molecules include e.g., T cell receptors (TCRs) and antigen-binding fragments thereof, antibodies and antigen binding fragments thereof, and TCR-like CAR. They exhibit antigenic specificity for binding or recognizing such peptide epitopes. In some aspects, engineered cells that express a provided

binding molecule, e.g. a TCR or antigen-binding fragment, exhibit cytotoxic activity against target cells expressing the peptide epitope, such as cancer cells or cells that are infected with a virus (e.g., HPV or EBV).

In some aspects, the TCR, antigen binding fragments thereof, and TCR-derived binding molecules recognize or bind to epitopes in the context of an MHC molecule, such as an MHC Class I molecule or an MHC class II molecule. Both MHC Class I molecules or MHC class II molecules are human leukocyte antigens (HLA). They play an important component of adaptive immune system. The HLA expression is controlled by genes located on chromosome 6. It encodes cell surface molecules specialized to present antigenic peptides to the T-cell receptor on T cells.

In some embodiments, the TCR, antigen binding fragments thereof, and TCR-derived binding molecules recognize or bind to epitopes in the context of an MHC Class I molecule. The MHC Class I molecule is a human leukocyte antigen (HLA)-A2 molecule, including any one or more subtypes thereof, e.g. HLA-A*0201, *0202, *0203, *0206, or *0207. The human leukocyte antigen A2 (HLA-A2) is among the most common human serotypes. In some cases, there can be differences in the frequency of subtypes between different populations. For example, more than 95% of the HLA-A2 positive Caucasian population is HLA- A*0201, whereas in the Chinese population the frequency has been reported to be approximately 23% for HLA-A*0201, 45% for HLA-A*0207, 8% for HLA-A*0206 and 23% for HLA-A*0203. In some embodiments, the MHC molecule is HLA-A*0201.

In some embodiments, the binding molecule, e.g., TCR or antigen-binding fragment thereof or TCR-derived binding molecule, is isolated or purified, or is recombinant. In some aspects, the binding molecule, e.g., TCR or antigen-binding fragment thereof or TCR-derived binding molecule, is fully human. In some embodiments, the binding molecule is monoclonal. In some aspects, the binding molecule is a single chain. In other embodiments, the binding molecule contains two chains. In some embodiments, the binding molecule, e.g., TCR, antigen-binding fragment thereof or TCR-derived binding molecule, is expressed on the surface of a cell.

In some embodiments, the TCR, antigen-binding fragment thereof, or TCR-derived binding molecules specifically binds to a tumor-associated antigen, e.g., BCMA, CD19, CD22, CD30, CD33, CD56, CD123 (also known as IL-3R), CEA, IL13Ra2, ALPP, EBV-related antigens (e.g., LMP2), EGFR, EGFRvIII, GD2, GPC3, HER2, HPV-related antigens (e.g., E6 or

E7), MAGE antigens, Mesothelin, MUC-1, NY-ESO-1, PSCA, PSMA, ROR1, WT1, or Claudin 18.2.

The TCR, antigen-binding fragment thereof, or TCR-derived binding molecules can have a Va and a Vb, or a region that is similar to Va and a region that is similar to Vb. In some embodiments, the TCR binds to latent membrane protein 2 (LMP2) of Epstein-Barr virus (EBV). In some embodiments, the Va region comprises the amino acid sequence set forth in any of SEQ ID NO: 22, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Vb region comprises the amino acid sequence set forth in any of SEQ ID NO: 23, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Va region comprises the amino acid sequence set forth in any of SEQ ID NO: 24, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Vb region comprises the amino acid sequence set forth in any of SEQ ID NO: 25, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Va region comprises the amino acid sequence set forth in any of SEQ ID NO: 26, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Vb region comprises the amino acid sequence set forth in any of SEQ ID NO: 27, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto.

In some embodiments, the TCR binds to tumor antigen E6 of human papilloma virus (HPV). In some embodiments, the Va region comprises the amino acid sequence set forth in any of SEQ ID NO: 28, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Vb region comprises the amino acid sequence set forth in any of SEQ ID NO: 29, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Va region comprises the amino acid sequence set forth in any of SEQ ID NO: 32, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Vb region comprises the amino acid sequence set forth in any of SEQ ID

NO: 33, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto.

In some embodiments, the TCR binds to tumor antigen E7 of human papilloma virus (HPV). In some embodiments, the Va region comprises the amino acid sequence set forth in any of SEQ ID NO: 30, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Vb region comprises the amino acid sequence set forth in any of SEQ ID NO: 31, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto.

In some embodiments, the TCR binds to NY-ESO-1 (Cancer/testis antigen 1, also known as LAGE2). In some embodiments, the Va region comprises the amino acid sequence set forth in any of SEQ ID NO: 34, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the Vb region comprises the amino acid sequence set forth in any of SEQ ID NO: 35, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto.

In some embodiments, the Va region comprises one or more Va CDR sequences as described herein. In some embodiments, the Vb region comprises one or more Vb CDR sequences as described herein.

In some embodiments, the nucleic acid encoding the alpha chain and the nucleic acid encoding the beta chain can be connected via a linker, such as any described elsewhere herein.

In some embodiments, by binding to the antigen of interest, the TCR or antigen-binding fragment thereof, or TCR-derived binding molecules, can activate T cells (e.g., by activating TCR signaling pathway). In some embodiments, the activation can upregulate immune response, increase expression of cytokines (e.g., IFN γ) and/or CD107a, promote T-cell proliferation and T cell mediated killing.

In some embodiments, the TCR or antigen-binding fragment thereof, or TCR-derived binding molecules as described herein can increase immune response, activity or number of T cells by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2 folds, 3 folds, 5 folds, 10 folds, or 20 folds. In some embodiments, the TCR or antigen-binding fragment thereof, or TCR-derived binding molecules, when the antigen of interest is present, can increase serum

concentrations of IFN- γ . In some embodiments, the activation can induce at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 5 folds, 10 folds, 100 folds, or 1000 folds increase of the serum concentrations of IFN- γ . In some embodiments, the activation can induce at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 3 folds, 4 folds, or 5 folds increase of specific killing of target cells.

In some aspects, the provided recombinant TCRs include TCRs that are at least partially CD8-independent. In some aspects, the provided recombinant TCRs include TCRs that are at least partially CD8-dependent.

In some embodiments, the TCR or antigen-binding fragment thereof, or TCR-derived binding molecules have a relatively high expression efficiency. For example, the expression efficiency for the TCR or antigen-binding fragment thereof, or TCR-derived binding molecules described herein can be at least 10%, 20%, 30%, 40%, 50%, or 100% higher than an reference molecule (e.g., an endogenous TCR) under the same conditions.

In some embodiments, the binding molecule, e.g. TCR, does not exhibit cross-reactive or off-target binding, such as undesirable off-target binding, e.g. off-target binding to antigens present in healthy or normal tissues or cells.

CHIMERIC ANTIGEN RECEPTORS AND BINDING MOLECULES

Chimeric antigen receptors (CARs) combine many facets of normal T cell activation into a single protein. They link an extracellular antigen recognition domain to an intracellular signaling domain, which activates the T cell when an antigen is bound. CARs are typically composed of four regions: an antigen recognition domain, an extracellular hinge region, a transmembrane domain, and an intracellular T-cell signaling domain.

The antigen recognition domain is exposed to the outside of the cell, in the ectodomain portion of the receptor. It interacts with potential target molecules and is responsible for targeting the CAR-T cell to any cell expressing a matching molecule. The antigen recognition domain is typically derived from the variable regions of a monoclonal antibody linked together as a single-chain variable fragment (scFv). An scFv is a chimeric protein made up of the light (VL) and heavy (VH) chains of immunoglobulins, connected with a short linker peptide. The linker between the two chains consists of hydrophilic residues with stretches of glycine and serine in it for flexibility as well as stretches of glutamate and lysine for added solubility. In some

embodiments, the antigen binding domain specifically binds to a tumor associated antigen, e.g., BCMA, CD19, CD22, CD30, CD33, CD56, CD123 (also known as IL-3R), CEA, EBV-related antigens (e.g., LMP2), EGFR, GD2, GPC3, HER2, HPV-related antigens (e.g., E6), MAGE antigens, Mesothelin, MUC-1, NY-ESO-1, PSCA, PSMA, ROR1, WT1, or Claudin 18.2.

The hinge, also called a spacer, is a small structural domain that sits between the antigen recognition region and the cell's outer membrane. An ideal hinge enhances the flexibility of the scFv receptor head, reducing the spatial constraints between the CAR and its target antigen. This promotes antigen binding and synapse formation between the CAR-T cells and target cells. Hinge sequences are often based on membrane-proximal regions from other immune molecules including IgG, CD8, and CD28.

The transmembrane domain is a structural component, consisting of a hydrophobic alpha helix that spans the cell membrane. It anchors the CAR to the plasma membrane, bridging the extracellular hinge and antigen recognition domains with the intracellular signaling region. This domain is essential for the stability of the receptor as a whole. Generally, the transmembrane domain from the most membrane-proximal component of the endodomain is used, but different transmembrane domains result in different receptor stability. The CD28 transmembrane domain is known to result in a highly expressed, stable receptor. In some embodiments, the transmembrane domain is a transmembrane domain of 4-1BB/CD137, an alpha chain of a T cell receptor, a beta chain of a T cell receptor, CD3 epsilon, CD4, CD5, CD8 alpha, CD9, CD16, CD19, CD22, CD33, CD37, CD45, CD64, CD80, CD86, CD134, CD137, CD154, or a zeta chain of a T cell receptor, or any combination thereof.

The intracellular T-cell signaling domain lies in the receptor's endodomain, inside the cell. After an antigen is bound to the external antigen recognition domain, CAR receptors cluster together and transmit an activation signal. Then the internal cytoplasmic end of the receptor perpetuates signaling inside the T cell. Normal T cell activation relies on the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic domain of CD3-zeta. To mimic this process, CD3-zeta's cytoplasmic domain is commonly used as the main CAR endodomain component. Other ITAM-containing domains have also been tried, but are not as effective. T cells also require co-stimulatory molecules in addition to CD3 signaling in order to persist after activation. For this reason, the endodomains of CAR receptors typically also include one or more chimeric domains from co-stimulatory proteins. Signaling domains from a

wide variety of co-stimulatory molecules have been successfully tested, including CD28, CD27, CD134 (OX40), and CD137 (4-1BB).

Various CAR molecules and vectors expressing these CAR molecules can be used in the methods described herein. In some embodiments, the CAR molecules specifically binds to a tumor-associated antigen, e.g., BCMA. In some embodiments, the CAR comprises the amino acid sequence set forth in any of SEQ ID NO: 37, 38, or 39, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the CAR molecules specifically binds to CD19. In some embodiments, the CAR comprises the amino acid sequence set forth in any of SEQ ID NO: 36, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the CAR molecules specifically targets to IL13Ra2. In some embodiments, the CAR comprises the amino acid sequence set forth in any of SEQ ID NO: 40, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. Some of these CAR molecules or target binding domains thereof are described e.g., WO2018200496A1, WO2019241686A1, WO2018085690A1, WO2018028647A1, and WO2018052828A1; each of which is incorporated herein by reference in its entirety.

Exemplary antigen receptors, including CARs, and methods for engineering and introducing such receptors into cells, include those described, for example, in Chandran *et al.*, "T cell receptor-based cancer immunotherapy: Emerging efficacy and pathways of resistance." Immunological reviews 290.1 (2019): 127-147; Cartellieri, Marc, *et al.*, "Chimeric antigen receptor-engineered T cells for immunotherapy of cancer." BioMed Research International 2010 (2010); and PCT publication No. WO2017173256A1; US2002/131960, US2013/287748, US2013/0149337, U.S. 6,451,995, U.S. 7,446,190, U.S. 8,252,592; each of which is incorporated herein by reference in its entirety.

In some embodiments, the CAR described herein comprises an antibody mimetic. Antibody mimetics are compounds that behave in a similar fashion to antibodies, and bind to specific antigens, but not related to antibodies (e.g., antibody fragments). In some embodiments, the antibody mimetic is a peptide, a nucleic acid, a small molecule, or combinations thereof. In some embodiments, the antibody mimetic is an affibody molecule (e.g., Z domain of Protein A), an adnectin, a monobody (e.g., 10th type III domain of fibronectin), a peptide aptamer, a peptide

affimer (e.g., cystatin), an affilin (e.g., gamma-B crystallin or ubiquitin), an affitin (e.g., Sac7d), an alphabody (e.g., triple helix coiled coil), an anticalin (e.g., a lipocalin), an avimer (e.g., A domains of various membrane receptors), a fynomer (e.g., SH3 domain of Fyn), an armadillo repeat protein, a DARPin (e.g., an ankyrin repeat motif), a Kunitz domain peptide (e.g., Kunitz domains of various protease inhibitors), a designed ankyrin repeat protein, a nanoCLAMP (e.g., carbohydrate binding module 32-2), a knottin, or combinations thereof. Detailed description can be found, e.g., in Yu *et al.*, "Beyond antibodies as binding partners: the role of antibody mimetics in bioanalysis." *Annual Review of Analytical Chemistry* 10 (2017): 293-320; and Ta and Brian, "Antibody and antibody mimetic immunotherapeutics." (2017): 1301-1304; each of which is incorporated herein by reference in its entirety.

ENGINEERED CELLS

The present disclosure provides engineered cells (e.g., immune cells, T cells, NK cells, tumor-infiltrating lymphocytes) that express IL-12 (e.g., membrane-tethered IL-12), TCR, CAR, and/or various proteins as described herein. These engineered cells can be used to treat various disorders or disease as described herein (e.g., virus infection, cancers, virus-induced disorders).

In various embodiments, the cell that is engineered can be obtained from e.g., humans and non-human animals. In various embodiments, the cell that is engineered can be obtained from bacteria, fungi, humans, rats, mice, rabbits, monkeys, pig or any other species. Preferably, the cell is from humans, rats or mice. In some embodiments, the cells are mouse lymphocytes and engineered (e.g., transduced) to express the TCR, CAR, or antigen-binding fragment thereof. In some embodiments, the cell is obtained from humans. In various embodiments, the cell that is engineered is a blood cell. Preferably, the cell is a leukocyte (e.g., a T cell), lymphocyte or any other suitable blood cell type. In some embodiments, the cell is a peripheral blood cell. In some embodiments, the cell is a tumor-infiltrating lymphocyte (TIL). In some embodiments, the cell is a T cell, B cell or NK cell. In some embodiments, the cells are human peripheral blood mononuclear cells (PBMCs). In some embodiments, the human PBMCs are CD3⁺ cells. In some embodiments, the human PBMCs are CD8⁺ cells.

In some embodiments, the cell is a T cell. In some embodiments, the T cells can express a cell surface receptor that recognizes a specific antigenic moiety on the surface of a target cell. The cell surface receptor can be a wild type or recombinant T cell receptor (TCR), a chimeric

antigen receptor (CAR), or any other surface receptor capable of recognizing an antigenic moiety that is associated with the target cell. T cells can be obtained by various methods known in the art, e.g., *in vitro* culture of T cells (e.g., tumor infiltrating lymphocytes) isolated from patients. Genetically modified T cells can be obtained by transducing T cells (e.g., isolated from the peripheral blood of patients), with a viral vector. In some embodiments, the T cell is a TCR gene-modified or CAR-modified T cell. In some embodiments, the T cells are CD4⁺ T cells, CD8⁺ T cells, or regulatory T cells. In some embodiments, the T cells are T helper type 1 T cells and T helper type 2 T cells. In some embodiments, the T cell expressing this receptor is an $\alpha\beta$ -T cell. In alternate embodiments, the T cell expressing this receptor is a $\gamma\delta$ -T cell. In some embodiments, the T cells are central memory T cells. In some embodiments, the T cells are effector memory T cells. In some embodiments, the T cells are naïve T cells.

In some embodiments, the cell is an NK cell. In some embodiments, preparation of the engineered cells includes one or more culture and/or preparation steps. The cells for introduction of the binding molecule, e.g., TCR or CAR, can be isolated from a sample, such as a biological sample, e.g., one obtained from or derived from a subject. In some embodiments, the subject from which the cell is isolated is one having the disease or condition or in need of a cell therapy or to which cell therapy will be administered. The subject in some embodiments is a human in need of a particular therapeutic intervention, such as the adoptive cell therapy for which cells are being isolated, processed, and/or engineered.

In some embodiments, the cells are stem cells, such as multipotent and pluripotent stem cells, including induced pluripotent stem cells (iPSCs). The cells can be primary cells, such as those isolated directly from a subject and/or isolated from a subject and frozen. In some embodiments, the stem cells are cultured with additional differentiation factors to obtain desired cell types (e.g., T cells).

Different cell types can be obtained from appropriate isolation methods. The isolation methods include the separation of different cell types based on the expression or presence in the cell of one or more specific molecules, such as surface markers, e.g., surface proteins, intracellular markers, or nucleic acid. In some embodiments, any known method for separation based on such markers can be used. In some embodiments, the separation is affinity- or immunoaffinity-based separation. For example, the isolation in some aspects includes separation of cells and cell populations based on the cells' expression or expression level of one or more

markers, typically cell surface markers, for example, by incubation with an antibody or binding partner that specifically binds to such markers, followed generally by washing steps and separation of cells having bound the antibody or binding partner, from those cells having not bound to the antibody or binding partner.

Such separation steps can be based on positive selection, in which the cells having bound the reagents are retained for further use, and/or negative selection, in which the cells having not bound to the antibody or binding partner are retained. In some examples, both fractions are retained for further use. In some aspects, negative selection can be particularly useful where no antibody is available that specifically identifies a cell type in a heterogeneous population, such that separation is best carried out based on markers expressed by cells other than the desired population.

Also provided are methods, nucleic acids, compositions, and kits, for expressing the binding molecules, and for producing the genetically engineered cells expressing such binding molecules. The genetic engineering generally involves introduction of a nucleic acid encoding the therapeutic molecule, e.g. TCR, CAR, e.g. TCR-like CAR, IL-12 (e.g., membrane-tethered IL-12), polypeptides, fusion proteins, into the cell, such as by retroviral transduction, transfection, or transformation. In some embodiments, gene transfer is accomplished by first stimulating the cell, such as by combining it with a stimulus that induces a response such as proliferation, survival, and/or activation, e.g., as measured by expression of a cytokine or activation marker, followed by transduction of the activated cells, and expansion in culture to numbers sufficient for clinical application.

In some embodiments, recombinant nucleic acids are transferred into cells using recombinant infectious virus particles, such as, e.g., vectors derived from simian virus 40 (SV40), adenoviruses, adeno-associated virus (AAV). In some embodiments, recombinant nucleic acids are transferred into T cells using recombinant lentiviral vectors or retroviral vectors, such as gamma-retroviral vectors. In some embodiments, the retroviral vector has a long terminal repeat sequence (LTR), e.g., a retroviral vector derived from the Moloney murine leukemia virus (MoMLV), myeloproliferative sarcoma virus (MPSV), murine embryonic stem cell virus (MESV), murine stem cell virus (MSCV), or spleen focus forming virus (SFFV). Most retroviral vectors are derived from murine retroviruses. In some embodiments, the retroviruses include those derived from any avian or mammalian cell source. The retroviruses typically are

amphotropic, meaning that they are capable of infecting host cells of several species, including humans. In some embodiments, the vector is a lentivirus vector. In some embodiments, recombinant nucleic acids are transferred into T cells via electroporation. In some embodiments, recombinant nucleic acids are transferred into T cells via transposition. Other methods of introducing and expressing genetic material in immune cells include calcium phosphate transfection, protoplast fusion, cationic liposome-mediated transfection; tungsten particle-facilitated microparticle bombardment and strontium phosphate DNA co-precipitation. Many of these methods are described e.g., in WO2019195486, which is incorporated herein by reference in its entirety.

In some aspects, development of a humanized and/or fully human recombinant TCR presents technical challenges. For example, in some aspects, a humanized and/or a fully human recombinant TCR receptor, when engineered into a human T cell, may compete with endogenous TCR complexes and/or can form mispairings with endogenous TCRA and/or TCRB chains, which may, in certain aspects, reduce recombinant TCR signaling, activity, and/or expression, and ultimately result in reduced activity of the engineered cells. The engineered cell can be genetically modified. In some embodiments, the engineered cells can comprise a genetic disruption of a T cell receptor alpha constant (TRAC) gene and/or a T cell receptor beta constant (TRBC) gene. In some embodiments, the TRBC gene is one or both of a T cell receptor beta constant 1 (TRBC1) or T cell receptor beta constant 2 (TRBC2) gene. In some embodiments, the engineered cells do not express endogenous TCR a chain and/or TCR b chain. In some other aspects, non-human constant domains are used, e.g., rodent (e.g., mouse) constant domains. The use of non-human constant domains can effectively reduce the likelihood of mispairing.

Also provided are populations of engineered cells, compositions containing such cells and/or enriched for such cells, such as in which cells expressing the binding molecule make up at least 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more percent of the total cells in the composition or cells of a certain type such as T cells, CD8⁺ or CD4⁺ cells.

In some embodiments, the engineered cells (e.g. TCR-T cells) are co-cultured with target cells (e.g., antigen presenting cells) for at least or about 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 18 hours, 1 day, 2 days, 3 days, or longer, such that the engineered cells (e.g., TCR-T cells) can be activated. In some embodiments, the target cells are Jurkat cells. In some

embodiments, the target cells are LLC-HLA-A2-Peplinker (LLW) melanoma cells. In some embodiments, the target cells are antigen-presenting cells. In some embodiments, the target cells express major histocompatibility complex (MHC) (e.g., class I, class II, and/or class III MHC). In some embodiments, the target cells comprise human leukocyte antigen (HLA) system.

In some embodiments, IL-12 and modified IL-12 can be expressed by the engineered cells. For example, the fusion protein comprising the modified IL-12 described herein can be expressed on cell surface of engineered cells, e.g., when the fusion protein is a membrane-tethered protein. In some instances, the fusion protein comprising modified IL-12 described herein can be expressed and secreted, e.g., when the fusion protein is a soluble protein.

The expression of IL-12 in the engineered cells provides some additional benefits. For example, it can increase production of IFN- γ , which is the most potent mediator of IL-12 actions, from NK and T cells, stimulate of growth and cytotoxicity of activated NK cells, CD8+ and CD4+ T cells, shift differentiation of CD4+ Th0 cells toward the Th1 phenotype, increase antibody-dependent cellular cytotoxicity (ADCC) against tumor cells, and induce IgG and suppression of IgE production from B cells, e.g., by at least or about 1 fold, 2 folds, 3 folds, 4 folds, 5 folds, 10 folds, or 20 folds.

In some embodiments, co-culturing with the target cells can increase cytokine (e.g., IFN γ) secretion of the engineered cells by at least or about 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 30 folds, 40 folds, 50 folds, 60 folds, 70 folds, 80 folds, 90 folds, 100 folds, 200 folds, 500 folds, 1000 folds, 2000 folds, 5000 folds, 10000 folds, or more as compared to the cytokine secretion level of the engineered cell without co-culturing.

In some embodiments, modified IL-12 expression can increase cytokine (e.g., IFN γ) expression or secretion of the engineered cells (e.g., TCR-T cells) by at least or about 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, 200 folds, 500 folds, 1000 folds, 2000 folds, 5000 folds, 10000 folds, or more as compared to the cytokine expression or secretion level of an engineered cell without expressing the modified IL-12. In some embodiments, modified IL-12 expression in the engineered cells (e.g., TCR-T cells) can stimulate cytokine (e.g., IFN γ) expression or secretion of immune cells in the vicinity of engineered cells by at least or about 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, 200 folds, 500 folds, 1000 folds, 2000 folds, 5000 folds, 10000 folds, or more as compared to the cytokine expression or secretion level of cells in the vicinity of the engineered cells that do not express the modified IL-12. In

some embodiments, modified IL-12 expression can increase expression of one or more early TCR activation markers (e.g., CD69) of the engineered cells (e.g., TCR-T cells) or immune cells in the vicinity of engineered cells by at least or about 1 fold, 2 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, 200 folds, 500 folds, 1000 folds, or more as compared to the expression levels of the one or more early TCR activation markers of an engineered cell that does not express the modified IL-12 or immune cells in the vicinity of engineered cells that does not express the modified IL-12.

In some embodiments, the cells are human PBMCs and engineered (e.g., transduced) to express the IL-12, TCR, CAR, or antigen-binding fragment thereof. In some embodiments, the engineered cells can further express the modified IL-12 as described herein. In some embodiments, the modified IL-12 is tethered to the membrane of the engineered cells. In some embodiments, when the engineered cells (e.g., PBMCs, TCRb⁺ PBMCs, or TCRb⁻ PBMCs) are co-cultured with target cells (e.g., antigen-presenting cells), the membrane-tethered IL-12 can increase cytokine (e.g., IFN γ) expression or secretion of the engineered cells by at least or about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 3 folds, 4 folds, 5 folds, 10 folds, 20 folds, 30 folds, 40 folds, 50 folds, 60 folds, 70 folds, 80 folds, 90 folds, 100 folds, or more as compared to the cytokine expression or secretion level of the engineered cells without expressing the membrane-tethered IL-12. In some embodiments, when the engineered cells (e.g., PBMCs or TCRb⁻ PBMCs) are co-cultured with target cells (e.g., antigen-presenting cells), the membrane-tethered IL-12 can increase activated T cell population by at least or about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 3 folds, 4 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, or more as compared to the activated T cell population in the engineered cells without expressing the membrane-tethered IL-12. In some embodiments, the T cell activation status can be measured by CD69 expression levels.

In some embodiments, the membrane-tethered IL-12 can increase CD4⁺ T cell population in the engineered cells (e.g., PBMCs) by at least or about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 3 folds, 4 folds, 5 folds, 10 folds, 20 folds, 50 folds, 100 folds, or more as compared to the CD4⁺ T cell population in the engineered cells without expressing the membrane-tethered IL-12. In some embodiments, the membrane-tethered IL-12 can decrease CD8⁺ T cell population in the engineered cells (e.g., PBMCs) to less than or about 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, or less as compared to the CD8⁺ T cell population

in the engineered cells without expressing the membrane-tethered IL-12.

In some embodiments, the membrane-tethered IL-12 can increase effector memory cell population in the engineered cells (e.g., CD4⁺ PBMCs or CD8⁺ PBMCs) by at least or about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1 fold, 2 folds, 3 folds, 4 folds, 5 folds, 6 folds, 7 folds, 8 folds, 9 folds, 10 folds, 20 folds, 50 folds, 100 folds, or more as compared to the effector memory cell population in the engineered cells without expressing the membrane-tethered IL-12. In some embodiments, the membrane-tethered IL-12 can decrease central memory cell population in the engineered cells (e.g., CD4⁺ PBMCs or CD8⁺ PBMCs) to less than or about 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, or less as compared to the central memory cell population in the engineered cells without expressing the membrane-tethered IL-12.

In some embodiments, the cells (e.g., PBMCs) are engineered to express the membrane-tethered IL-12 fusion protein comprising a soluble portion. In some embodiments, the soluble portion comprises the IL-12A and/or the IL-12B. In some embodiments, the soluble portion comprises a sequence that is at least or about 50%, 60%, 70%, 80%, 90%, or 100% identical to SEQ ID NO: 8. In some embodiments, the membrane-tethered IL-12 fusion protein cannot be released from the cells. In some embodiments, the IL-12 level in the medium from the cells expressing the membrane-tethered IL-12 fusion protein is less than 10%, 5%, 4%, 3%, 2%, 1%, or less as compared to cells that are engineered to express a soluble IL-12. In some embodiments, the concentration of IL-12 in the medium as detected by ELISA is less than 300, 200, or 100 pg/ml.

RECOMBINANT VECTORS

The present disclosure also provides recombinant vectors (e.g., an expression vectors) that include an isolated polynucleotide disclosed herein (e.g., a polynucleotide that encodes a polypeptide disclosed herein), host cells into which are introduced the recombinant vectors (i.e., such that the host cells contain the polynucleotide and/or a vector comprising the polynucleotide), and the production of recombinant polypeptides or fragments thereof by recombinant techniques.

A vector is a construct capable of delivering one or more polynucleotide(s) of interest to a host cell when the vector is introduced to the host cell. An “expression vector” is capable of delivering and expressing the one or more polynucleotide(s) of interest as an encoded polypeptide in a host cell into which the expression vector has been introduced. Thus, in an

expression vector, the polynucleotide of interest is positioned for expression in the vector by being operably linked with regulatory elements such as a promoter, enhancer, and/or a poly-A tail, either within the vector or in the genome of the host cell at or near or flanking the integration site of the polynucleotide of interest such that the polynucleotide of interest will be translated in the host cell introduced with the expression vector.

A vector can be introduced into the host cell by methods known in the art, e.g., electroporation, chemical transfection (e.g., DEAE-dextran), transformation, transfection, and infection and/or transduction (e.g., with recombinant virus). Thus, non-limiting examples of vectors include viral vectors (which can be used to generate recombinant virus), naked DNA or RNA, plasmids, cosmids, phage vectors, and DNA or RNA expression vectors associated with cationic condensing agents.

The present disclosure provides a recombinant vector comprising a nucleic acid construct suitable for genetically modifying a cell, which can be used for treatment of pathological disease or condition.

Any vector or vector type can be used to deliver genetic material to the cell. These vectors include but are not limited to plasmid vectors, viral vectors, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), and human artificial chromosomes (HACs). Viral vectors can include but are not limited to recombinant retroviral vectors, recombinant lentiviral vectors, recombinant adenoviral vectors, foamy virus vectors, recombinant adeno-associated viral (AAV) vectors, hybrid vectors, and plasmid transposons (e.g., sleeping beauty transposon system, and PiggyBac transposon system) or integrase based vector systems. Other vectors that are known in the art can also be used in connection with the methods described herein.

In some embodiments, the vector is a viral vector. The viral vector can be grown in a culture medium specific for viral vector manufacturing. Any suitable growth media and/or supplements for growing viral vectors can be used in accordance with the embodiments described herein. In some embodiments, a MP71 vector is used.

In some embodiments, the vector used is a recombinant retroviral vector. A retroviral vector is capable of directing the expression of a nucleic acid molecule of interest. A retrovirus is present in the RNA form in its viral capsule and forms a double-stranded DNA intermediate when it replicates in the host cell. Similarly, retroviral vectors are present in both RNA and

double-stranded DNA forms. The retroviral vector also includes the DNA form which contains a recombinant DNA fragment and the RNA form containing a recombinant RNA fragment. The vectors can include at least one transcriptional promoter/enhancer, or other elements which control gene expression. Such vectors can also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used. Long terminal repeats (LTRs) are identical sequences of DNA that repeat many times (e.g., hundreds or thousands of times) found at either end of retrotransposons or proviral DNA formed by reverse transcription of retroviral RNA. They are used by viruses to insert their genetic material into the host genomes. Optionally, the vectors can also include a signal which directs polyadenylation, selectable markers such as Ampicillin resistance, Neomycin resistance, TK, hygromycin resistance, phleomycin resistance histidinol resistance, or DHFR, as well as one or more restriction sites and a translation termination sequence. For example, such vectors can include a 5' LTR, a leading sequence, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof. Additionally, retroviral vector used herein can also refers to the recombinant vectors created by removal of the retroviral gag, pol, and env genes and replaced with the gene of interest.

In some embodiments, the vector or construct can contain a single promoter that drives the expression of one or more nucleic acid molecules. In some embodiments, such promoters can be multicistronic (bicistronic or tricistronic). For example, in some embodiments, transcription units can be engineered as a bicistronic unit containing an IRES (internal ribosome entry site), which allows coexpression of gene products (e.g. encoding an alpha chain and/or beta chain of a TCR and a modified IL-12) by a message from a single promoter. Alternatively, in some cases, a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three genes (e.g. encoding an alpha chain and/or beta chain of a TCR) separated from one another by sequences encoding a self-cleavage peptide (e.g., P2A or T2A) or a protease recognition site (e.g., furin). The ORF thus encodes a single polyprotein, which, either during (in the case of 2A e.g., T2A) or after translation, is cleaved into the individual proteins. In some cases, the peptide, such as T2A, can cause the ribosome to skip (ribosome skipping) synthesis of a peptide bond at the C-terminus of a 2A element, leading to separation between the end of the 2A sequence and the next peptide downstream.

Various cell lines can be used in connection with the vectors as described herein.

Exemplary eukaryotic cells that may be used to express polypeptides include, but are not limited to, COS cells, including COS 7 cells; 293 cells, including 293-6E cells; CHO cells, including CHO-S, DG44, Lec13 CHO cells, and FUT8 CHO cells; PER.C6® cells; and NSO cells. In some embodiments, a particular eukaryotic host cell is selected based on its ability to make desired post-translational modifications to the binding molecule. For example, in some embodiments, CHO cells produce polypeptides that have a higher level of sialylation than the same polypeptide produced in 293 cells. In one aspect, the disclosure relates to a cell comprising the vector or the pair of vectors as described herein. In some embodiments, the cell is a T cell.

In some cases, certain TCRs, may exhibit poor expression or activity in part due to mispairing and/or competition with endogenous TCR chains and/or other factors. One method to address these challenges has been to design recombinant TCRs with mouse constant domains to prevent mispairings with endogenous human TCR α or β chains. However, the use of recombinant TCRs with mouse sequences may present a risk for immune response. In some embodiments, a genetic disruption is introduced, e.g., by gene editing, at an endogenous gene encoding one or more TCR chains.

As shown in FIGS. 1A-1B, provided herein are mouse and human IL-12 expression vectors comprising nucleic acid sequences encoding IL-12B (p40) and IL-12A (p35). In some embodiments, the nucleic acid sequences encoding the IL-12B and IL-12A are linked by a linker sequence. In some embodiments, the vectors further comprise a sequence encoding an immunoglobulin hinge region, two or three constant domains (e.g., one or two CH2, and a CH3), and a transmembrane region (e.g., a CD4 transmembrane region), linked after IL-12A.

In some embodiments, the vector encoding IL-12 further comprises a sequence encoding a heavy chain variable region (vH) and light chain variable region (vL) of a tumor-targeting antibody (e.g., NHS-76) before IL-12B. In some embodiments, the nucleic acid sequences encoding the vH and vL are linked by a linker sequence. In some embodiments, the nucleic acid sequences encoding the vL and IL-12B are linked by a linker sequence.

In some embodiments, the vector described herein comprises a sequence encoding a signal peptide sequence. In some embodiments, the sequence encoding the signal peptide sequence is linked to the sequence encoding IL-12B. In some embodiments, the sequence encoding the signal peptide sequence is linked to the sequence encoding vH of the tumor-targeting antibody. In some embodiments, the vector further comprises a sequence encoding a

linker peptide sequence (e.g., P2A) before the signal peptide sequence.

As shown in FIGS. 1C-1D, in some embodiments, the vector described herein further comprises a sequence encoding a T cell receptor (TCR), or a chimeric antigen receptor (CAR). In some embodiments, the sequence encoding the TCR or the CAR is linked before the sequence encoding the signal peptide sequence (e.g., P2A).

The present disclosure also provides nucleic acids that encodes human IL-12 fusion proteins. In some embodiments, the nucleic acid that encodes the IL-12 fusion protein described herein is set forth in SEQ ID NO: 7, or a nucleic acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the nucleic acid that encodes the membrane-tethered IL-12 fusion protein described herein is set forth in SEQ ID NO: 9, or a nucleic acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the nucleic acid that encodes the membrane-tethered IL-12 fusion protein described herein is set forth in SEQ ID NO: 11, or a nucleic acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto. In some embodiments, the nucleic acid that encodes the soluble IL-12 fusion protein described herein is set forth in SEQ ID NO: 13, or a nucleic acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto.

The term “Linker” (L) or “linker domain” or “linker region” as used herein refer to an oligo- or polypeptide region from about 1 to 100 amino acids in length, which links together any of the domains/regions. Linkers can be composed of flexible residues like glycine and serine so that the adjacent protein domains are free to move relative to one another. Longer linkers can be used when it is desirable to ensure that two adjacent domains do not sterically interfere with one another. Linkers can be cleavable or non-cleavable. Examples of cleavable linkers include 2A linkers (for example P2A, T2A), 2A-like linkers or functional equivalents thereof and combinations thereof. In some embodiments, the linkers include the picornaviral 2A-like linker, CHYSEL sequences of porcine teschovirus (P2A), Thosea asigna virus (T2A) or combinations, variants and functional equivalents thereof. Other linkers will be apparent to those of skill in the art and can be used in the methods described herein.

In some embodiments, the linker peptide sequence comprises at least or about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, or 50 amino acid residues. In some

embodiments, the linker sequence comprises at least or about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 20, 25, 30, or 40 glycine residues. In some embodiments, the linker sequence comprises or consists of both glycine and serine residues. In some embodiments, the linker sequence comprises or consists of a sequence that is at least or about 70%, at least or about 75%, at least or about 80%, at least or about 85%, at least or about 90%, at least or about 95%, at least or about 99%, or 100% identical to SEQ ID NO: 17, GGGGSGGGGS (SEQ ID NO: 18), or GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 19). In some embodiments, the linker sequence comprises at least 1, 2, 3, 4, 5, or 6 repeats of GGGGS (SEQ ID NO: 20).

The present disclosure also provides a nucleic acid sequence comprising a nucleotide sequence encoding any of the IL-12, modified IL-12, CAR, TCRs, antigen binding fragments thereof, and/or TCR-derived binding molecules (including e.g., functional portions and functional variants thereof, polypeptides, or proteins described herein). “Nucleic acid” as used herein can include “polynucleotide,” “oligonucleotide,” and “nucleic acid molecule,” and generally means a polymer of DNA or RNA, which can be single-stranded or double-stranded, synthesized or obtained from natural sources, which can contain natural, non-natural or altered nucleotides. Furthermore, the nucleic acid comprises complementary DNA (cDNA). It is generally preferred that the nucleic acid does not comprise any insertions, deletions, inversions, and/or substitutions. However, it can be suitable in some instances, as discussed herein, for the nucleic acid to comprise one or more insertions, deletions, inversions, and/or substitutions.

The nucleic acids as described herein can be constructed based on chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. For example, a nucleic acid can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides. In some of any such embodiments, the nucleotide sequence is codon-optimized.

The present disclosure also provides the nucleic acids comprising a nucleotide sequence complementary to the nucleotide sequence of any of the nucleic acids described herein or a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of any of the nucleic acids described herein.

In some embodiments, the nucleotide sequence encoding the alpha chain and the nucleotide sequence encoding the beta chain are separated by a peptide sequence that causes ribosome skipping. In some embodiments, the peptide that causes ribosome skipping is a P2A or T2A peptide. In some embodiments, the nucleic acid is synthetic. In some embodiments, the

nucleic acid is cDNA.

In some embodiments, the vector can additionally include a nucleic acid sequence that encodes a checkpoint inhibitor (CPI) (e.g., an inhibitory protein). In some embodiments, the checkpoint inhibitor is e.g., any antibody or antigen binding fragment thereof as described herein. In some embodiments, the antibody or antigen binding fragments thereof can specifically bind to PD-1, PD-L1, PD-L2, 2B4 (CD244), 4-1BB, A2aR, B7.1, B7.2, B7-H2, B7-H3, B7-H4, B7-H6, BTLA, butyrophilins, CD160, CD48, CTLA4, GITR, gp49B, HHLA2, HVEM, ICOS, ILT-2, ILT-4, KIR family receptors, LAG-3, OX-40, PIR-B, SIRPalpha (CD47), TFM-4, TIGIT, TIM-1, TIM-3, TIM-4, or VISTA. In some embodiments, the inhibitory protein is a scFv (e.g., an anti-PD-1 scFv). In some embodiments, the vector can additionally include a nucleic acid sequence that encodes a bifunctional trap fusion protein. In some embodiments, the bifunctional trap protein targets both the PD-1 and TGF- β . In some embodiments, the bifunctional trap protein targets both the PD-L1 and TGF- β . In some embodiments, the bifunctional fusion protein designed to block PD-L1 and sequester TGF- β . M7824 (MSB0011395C) comprises the extracellular domain of human TGF- β receptor II (TGF β RII) linked to the C-terminus of the human anti-PD-L1 scFv, based on the human IgG1 monoclonal antibody (mAb) avelumab. In some embodiments, the bifunctional fusion protein comprises the extracellular domain of human TGF- β receptor II (TGF β RII) linked to the C-terminus of the human anti-PD-1 scFv.

In some of any such embodiments, the IL-12 (e.g., membrane-tethered IL-12), CAR, TCR or antigen-binding fragment thereof is encoded by a nucleotide sequence that has been codon-optimized. In certain embodiments, the polypeptide comprises a signal peptide. In some of any such embodiments, the polypeptide and/or the fusion protein is recombinant.

The disclosure also provides a nucleic acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any nucleotide sequence as described herein, and an amino acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any amino acid sequence as described herein. In some embodiments, the disclosure relates to nucleotide sequences encoding any peptides that are described herein, or any amino acid sequences that are encoded by any nucleotide sequences as described herein.

In some embodiments, the nucleic acid sequence is at least or about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 150, 200, 250, 300, 350, 400, 500, or 600 nucleotides. In some embodiments, the amino acid sequence is at least or about 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 600, 700, 800, or 900 amino acid residues. In some embodiments, the nucleic acid sequence is less than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 150, 200, 250, 300, 350, 400, 500, or 600 nucleotides. In some embodiments, the amino acid sequence is less than 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 600, 700, 800, or 900 amino acid residues.

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

METHOD FOR PREPARATION OF ENGINEERED CELLS

The present disclosure provides a method or process for preparing, manufacturing and/or using the engineered cells for treatment of pathological diseases or conditions.

The cells for introduction of the protein described herein, e.g., IL-12, TCR, and/or CAR, can be isolated from a sample, such as a biological sample, e.g., one obtained from or derived from a subject. In some embodiments, the subject from which the cell is isolated is one having the disease or condition or in need of a cell therapy or to which cell therapy will be administered. The subject in some embodiments is a human in need of a particular therapeutic intervention, such as the adoptive cell therapy for which cells are being isolated, processed, and/or engineered.

Accordingly, the cells in some embodiments are primary cells, e.g., primary human cells.

The samples include tissue, fluid, and other samples taken directly from the subject, as well as samples resulting from one or more processing steps, such as separation, centrifugation, genetic engineering (e.g. transduction with viral vector), washing, and/or incubation. The biological sample can be a sample obtained directly from a biological source or a sample that is processed. Biological samples include, but are not limited to, body fluids, such as blood, plasma, serum, cerebrospinal fluid, synovial fluid, urine and sweat, tissue and organ samples, including processed samples derived therefrom.

In some aspects, the sample from which the cells are derived or isolated is blood or a blood-derived sample, or is or is derived from an apheresis or 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, liver, lung, stomach, intestine, colon, kidney, pancreas, breast, bone, prostate, cervix, testes, ovaries, tonsil, or other organ, and/or cells derived therefrom. Samples include, in the context of cell therapy, e.g., adoptive cell therapy, samples from autologous and allogeneic sources.

In some embodiments, the cells are derived from cell lines, e.g., T cell lines. The cells in some embodiments are obtained from a xenogeneic source, for example, from mouse, rat, or non-human primate. In some embodiments, the cells are isolated from mouse lymph nodes.

In some embodiments, the blood cells collected from the subject are washed, e.g., to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In some embodiments, the cells are washed with phosphate buffered saline (PBS). In some embodiments, the wash solution lacks calcium and/or magnesium and/or many or all divalent cations. In some aspects, a washing step is accomplished a semi-automated "flow-through" centrifuge. In some aspects, a washing step is accomplished by tangential flow filtration (TFF). In some embodiments, the cells are resuspended in a variety of biocompatible buffers after washing, such as, for example, $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS. In certain embodiments, components of a blood cell sample are removed and the cells directly resuspended in culture media. In some embodiments, the methods include density-based cell separation methods, such as the preparation of white blood cells from peripheral blood by lysing the red blood cells and centrifugation through a Percoll or Ficoll gradient.

In some embodiments, the method comprises one or more steps of: e.g., isolating the T

cells from a patient's blood; transducing the population T cells with a viral vector including the nucleic acid construct encoding a genetically engineered antigen receptor; expanding the transduced cells *in vitro*; and/or infusing the expanded cells into the patient, where the engineered T cells will seek and destroy antigen positive tumor cells. In some embodiments, the nucleic acid construct further includes a sequence encoding an inhibitory protein. In some embodiments, these engineered T cells can block PD-1/PD-L1 immunosuppression and strengthen the antitumor immune response. The method further comprises: transfection of T cells with the viral vector containing the nucleic acid construct.

In some embodiments, the methods involve introducing any vectors described herein into a cell *in vitro* or *ex vivo*. In some embodiments, the vector is a viral vector and the introducing is carried out by transduction. In some embodiments, the cell is transduced for at least or about 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 18 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, or longer. In some embodiments, the methods further involve introducing into the cell one or more agent, wherein each of the one or more agent is independently capable of inducing a genetic disruption of a T cell receptor alpha constant (TRAC) gene and/or a T cell receptor beta constant (TRBC) gene. In some embodiments, the one or more agent is an inhibitory nucleic acid (e.g., siRNA). In some embodiments, the one or more agent is a fusion protein comprising a DNA-targeting protein and a nuclease or an RNA-guided nuclease (e.g., a clustered regularly interspaced short palindromic nucleic acid (CRISPR)-associated nuclease).

In some embodiments, the cell is a tumor infiltrating lymphocyte, and the cell is transfected with a vector encoding IL-12 or a modified IL-12. In some embodiments, the cell is a T cell, and the cell is transfected with a vector encoding IL-12 (e.g., a modified IL-12) and TCR or a vector encoding IL-12 (e.g., a modified IL-12) and CAR.

The transfection of T cells can be achieved by using any standard method such as calcium phosphate, electroporation, liposomal mediated transfer, microinjection, biolistic particle delivery system, or any other known methods by skilled artisan. In some embodiments, transfection of T cells is performed using the calcium phosphate method.

The present disclosure provides a method to create a personalized anti-tumor immunotherapy. Genetically engineered T cells can be produced from a patient's blood cells. These engineered T cells are then reinfused into the patient as a cellular therapy product.

Methods of preparing engineered cells and administering these engineered cells to a subject are known in the art, and are described e.g., in US Pat. No. 10,174,098 and Draper *et al.*, "Targeting of HPV-16+ Epithelial Cancer Cells By Tcr Gene Engineered t Cells Directed Against e6." Clinical Cancer Research 21.19 (2015): 4431-4439, both of which are incorporated by reference in their entirety.

METHODS OF TREATMENT

The methods disclosed herein can be used for various therapeutic purposes. In one aspect, the disclosure provides methods for treating a cancer in a subject, methods of reducing the rate of the increase of volume of a tumor in a subject over time, methods of reducing the risk of developing a metastasis, or methods of reducing the risk of developing an additional metastasis in a subject. In some embodiments, the treatment can halt, slow, retard, or inhibit progression of a cancer. In some embodiments, the treatment can result in the reduction of in the number, severity, and/or duration of one or more symptoms of the cancer in a subject.

In one aspect, the disclosure features methods that include administering a therapeutically effective amount of engineered cells expressing IL-12 (e.g., modified IL-12), TCR, CAR, antigen binding fragments thereof, and TCR-derived binding molecules to a subject in need thereof (e.g., a subject having, or identified or diagnosed as having, a cancer).

In some embodiments, the subject has a solid tumor (e.g., a heterogeneous solid tumor or a homogeneous solid tumor). In some embodiments, the subject has breast cancer (e.g., triple-negative breast cancer), carcinoid cancer, cervical cancer, endometrial cancer, glioma, head and neck cancer, liver cancer, lung cancer, small cell lung cancer, lymphoma, melanoma, ovarian cancer, pancreatic cancer, prostate cancer, renal cancer, colorectal cancer, gastric cancer, testicular cancer, thyroid cancer, bladder cancer, urethral cancer, or hematologic malignancy. In some embodiments, the cancer is unresectable melanoma or metastatic melanoma, non-small cell lung carcinoma (NSCLC), small cell lung cancer (SCLC), bladder cancer, or metastatic hormone-refractory prostate cancer. In some embodiment's, the cancer is cervical cancer, head and neck cancer, oropharyngeal cancers, anal cancer, penile cancer, vaginal cancer or vulvar cancer.

In some embodiments, the subject has a heterogeneous cancer. In some embodiments, the subject has a homogeneous cancer. In some embodiments, the heterogeneous cancer has at least

1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, or more cancer cells expressing different antigens as compared to one or more antigens that are expressed by the majority (e.g., at least 50%, 60%, 70%, 80%, or 90%) of cancer cells in the heterogeneous cancer. In some embodiments, the TCR, CAR, antigen binding fragments thereof, and/or TCR-derived binding molecules can bind to one or more antigens that are expressed by a fraction (e.g., at least 50%, 60%, 70%, 80%, 90%, 95%, or more) or all cells in the heterogeneous cancer. In some embodiments, the one or more antigens are not expressed by a fraction (e.g., less than 50%, 40%, 30%, 20%, 10%, or 5%) of cells in the heterogeneous cancer. In some embodiments, the one or more antigens are deactivated (e.g., cleaved, or through mechanisms such as immune escape), such that the TCR, CAR, antigen binding fragments thereof, and/or TCR-derived binding molecules can no longer recognize. In some embodiments, a fraction of cells (e.g., less than 50%, 40%, 30%, 20%, 10%, or 5%) in the heterogeneous cancer can express immune checkpoint molecules (e.g., PD-L1) to suppress immune response. Detailed descriptions of tumor antigen heterogeneity and antigen escape can be found, in O'Rourke *et al.*, "A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma." *Science translational medicine* 9.399 (2017): eaaa0984; Majzner and Crystal, "Tumor antigen escape from CAR T-cell therapy." *Cancer discovery* 8.10 (2018): 1219-1226; each of which is incorporated herein by reference in its entirety.

In some embodiments, the IL-12 (e.g., modified IL-12) expressed by the engineered cells described herein can provide improvement (e.g., killing cancer cells, or reducing tumor volume) of treating the heterogeneous cancer by at least or about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2 folds, 3 folds, 5 folds, 10 folds, 20 folds, 30 folds, 40 folds, 50 folds, 60 folds, 70 folds, 80 folds, 90 folds, 100 folds, or more as compared to similar engineered TCR or CAR cells that do not express the IL-12.

In some embodiments, the compositions and methods disclosed herein can be used for treatment of patients at risk for a cancer. Patients with cancer can be identified with various methods known in the art.

Furthermore, the disclosure provides methods for treating infection or infection associated conditions in a subject. In some embodiments, the treatment can halt, slow, retard, or inhibit progression of the disease. These methods generally involve administering a therapeutically effective amount of genetic engineered cells disclosed herein to a subject in need

thereof. In some embodiments, the disease or condition treated is an infectious disease or condition, such as, but not limited to, viral, retroviral, bacterial, and protozoal infections, immunodeficiency, Human Papilloma Virus (HPV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus, BK polyomavirus.

As used herein, by an "effective amount" is meant an amount or dosage sufficient to effect beneficial or desired results including halting, slowing, retarding, or inhibiting progression of a disease, e.g., a cancer. An effective amount will vary depending upon, e.g., an age and a body weight of a subject to which the therapeutic agent and/or therapeutic compositions is to be administered, a severity of symptoms and a route of administration, and thus administration can be determined on an individual basis.

As used herein, the term "delaying development of a disease" refers to defer, hinder, slow, retard, stabilize, suppress and/or postpone development of the disease (such as cancer). This delay can be of varying lengths of time, depending on the history of the disease and/or individual being treated. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop the disease. For example, a late stage cancer, such as development of metastasis, can be delayed.

An effective amount can be administered in one or more administrations. By way of example, an effective amount of a composition is an amount sufficient to ameliorate, stop, stabilize, reverse, inhibit, slow and/or delay progression of a cancer in a patient or is an amount sufficient to ameliorate, stop, stabilize, reverse, slow and/or delay proliferation of a cell (e.g., a biopsied cell, any of the cancer cells described herein, or cell line (e.g., a cancer cell line)) *in vitro*. As is understood in the art, an effective may vary, depending on, *inter alia*, patient history as well as other factors such as the type (and/or dosage) of compositions used.

Effective amounts and schedules for administrations may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage that must be administered will vary depending on, for example, the mammal that will receive the treatment, the route of administration, the particular type of therapeutic agents and other drugs being administered to the mammal. Guidance in selecting appropriate doses can be found in the literature. In addition, a treatment does not necessarily result in the 100% or complete treatment or prevention of a disease or a condition. There are multiple treatment/prevention methods available with a varying degree of therapeutic effect which one of

ordinary skill in the art recognizes as a potentially advantageous therapeutic mean.

In some aspects, the present disclosure also provides methods of diagnosing a disease/condition in a mammal, wherein the TCRs, CARs, antigen binding fragments, TCR-derived binding molecules interact with the sample(s) obtained from a subject to form a complex, wherein the sample can comprise one more cells, polypeptides, proteins, nucleic acids, antibodies, or antigen binding portions, blood, whole cells, lysates thereof, or a fraction of the whole cell lysates, e.g., a nuclear or cytoplasmic fraction, a whole protein fraction, or a nucleic acid fraction thereof, wherein the detection of the complex is the indicative of presence of a condition in the mammal, wherein the condition is cancer or infection. Further, the detection of the complex can be in any number of way known in the art but not limited to, ELISA, Flow cytometry, Fluorescence in situ hybridization (FISH), Polymerase chain reaction (PCR), microarray, southern blotting, electrophoresis, Phage analysis, chromatography and more. Thus, the treatment methods can further include determining whether a subject can benefit from a treatment as disclosed herein, e.g., by determining whether the subject has infection or cancer.

In any of the methods described herein, the engineered cells and, and/or at least one additional therapeutic agent can be administered to the subject at least once a week (e.g., once a week, twice a week, three times a week, four times a week, once a day, twice a day, or three times a day). In some embodiments, at least two different engineered cells (e.g., cells express different binding molecules) are administered in the same composition (e.g., a liquid composition). In some embodiments, engineered cells and at least one additional therapeutic agent are administered in the same composition (e.g., a liquid composition). In some embodiments, engineered cells and the at least one additional therapeutic agent are administered in two different compositions. In some embodiments, the at least one additional therapeutic agent is administered as a pill, tablet, or capsule. In some embodiments, the at least one additional therapeutic agent is administered in a sustained-release oral formulation.

In some embodiments, the one or more additional therapeutic agents can be administered to the subject prior to, concurrently with, or after administering the engineered cells to the subject.

In some embodiments, one or more additional therapeutic agents can be administered to the subject. The additional therapeutic agent can be a checkpoint inhibitor (CPI). In some embodiments, the checkpoint inhibitor is an inhibitory protein, e.g., an antibody or antigen

binding fragment thereof. The checkpoint inhibitor can inhibit or block one or more immune checkpoints, including e.g., PD-1, PD-L1, PD-L2, 2B4 (CD244), 4-1BB, A2aR, B7.1, B7.2, B7-H2, B7-H3, B7-H4, B7-H6, BTLA, butyrophilins, CD160, CD48, CTLA4, GITR, gp49B, HHLA2, HVEM, ICOS, ILT-2, ILT-4, KIR family receptors, LAG-3, OX-40, PIR-B, SIRPalpha (CD47), TFM-4, TIGIT, TIM-1, TIM-3, TIM-4, VISTA and combinations thereof. In some embodiments, the inhibitory protein blocks PD-1 or PD-L1. In various embodiments, the inhibitory protein comprises an anti-PD-1 scFv. The inhibitory protein is capable of leading to reduced expression of PD-1 or PD-L1 and/or inhibiting upregulation of PD-1 or PD-L1 in T cells in the population and/or physically obstructing the formation of the PD-1/PD-L1 complex and subsequent signal transduction. In some embodiments, the inhibitory protein blocks PD-1. In some embodiments, the additional therapeutic agent is an anti-OX40 antibody, an anti-PD-L1 antibody, an anti-PD-L2 antibody, an anti-LAG-3 antibody, an anti-TIGIT antibody, an anti-BTLA antibody, an anti-CTLA-4 antibody, or an anti-GITR antibody. In some embodiments, the additional therapeutic agent is an anti-CTLA4 antibody (e.g., ipilimumab), an anti-CD20 antibody (e.g., rituximab), an anti-EGFR antibody (e.g., cetuximab), an anti-CD319 antibody (e.g., elotuzumab), or an anti-PD1 antibody (e.g., nivolumab).

In some embodiments, the additional therapeutic agent is a bifunctional trap fusion protein. Bifunctional trap proteins can target both immune checkpoints and TGF- β negative regulatory pathways. In addition to expression of immune checkpoints, the tumor microenvironment contains other immunosuppressive molecules. Of particular interest is the cytokine TGF- β (TGFB), which has multiple functions in cancer. TGF- β prevents proliferation and promotes differentiation and apoptosis of tumor cells early in tumor development. However, during tumor progression, tumor TGF- β insensitivity arises due to the loss of TGF- β receptor expression or mutation to downstream signaling elements. TGF- β then promotes tumor progression through its effects on angiogenesis, induction of epithelial-to-mesenchymal transition (EMT), and immune suppression. High TGF- β serum level and loss of TGF- β receptor (TGF β R) expression on tumors correlates with poor prognosis. TGF β -targeted therapies have demonstrated limited clinical activity. In some embodiments, the bifunctional trap protein targets both the PD-1 and TGF- β . In some embodiments, the bifunctional trap protein targets both the PD-L1 and TGF- β . In some embodiments, the bifunctional fusion protein designed to block PD-L1 and sequester TGF- β . M7824 (MSB0011395C) comprises the extracellular domain of human

TGF- β receptor II (TGF β RII) linked to the C-terminus of the human anti-PD-L1 scFv, based on the human IgG1 monoclonal antibody (mAb) avelumab. In some embodiments, the bifunctional fusion protein comprises the extracellular domain of human TGF- β receptor II (TGF β RII) linked to the C-terminus of the human anti-PD-1 scFv. These bifunctional trap fusion proteins are described e.g., Knudson, *et al.*, "M7824, a novel bifunctional anti-PD-L1/TGF β Trap fusion protein, promotes anti-tumor efficacy as monotherapy and in combination with vaccine." *Oncoimmunology* 7.5 (2018): e1426519, which is incorporated herein by reference in its entirety. In some embodiments, the subject is treated by cells that express TCR or antigen-binding molecules as described herein and one or more bifunctional trap fusion proteins.

In some embodiments, the additional therapeutic agent can comprise one or more inhibitors selected from the group consisting of an inhibitor of B-Raf, an EGFR inhibitor, an inhibitor of a MEK, an inhibitor of ERK, an inhibitor of K-Ras, an inhibitor of c-Met, an inhibitor of anaplastic lymphoma kinase (ALK), an inhibitor of a phosphatidylinositol 3-kinase (PI3K), an inhibitor of an Akt, an inhibitor of mTOR, a dual PI3K/mTOR inhibitor, an inhibitor of Bruton's tyrosine kinase (BTK), and an inhibitor of Isocitrate dehydrogenase 1 (IDH1) and/or Isocitrate dehydrogenase 2 (IDH2). In some embodiments, the additional therapeutic agent is an inhibitor of indoleamine 2,3-dioxygenase-1 (IDO1) (e.g., epacadostat). In some embodiments, the additional therapeutic agent can comprise one or more inhibitors selected from the group consisting of an inhibitor of HER3, an inhibitor of LSD1, an inhibitor of MDM2, an inhibitor of BCL2, an inhibitor of CHK1, an inhibitor of activated hedgehog signaling pathway, and an agent that selectively degrades the estrogen receptor.

In some embodiments, the additional therapeutic agent can comprise one or more therapeutic agents selected from the group consisting of Trabectedin, nab-paclitaxel, Trebananib, Pazopanib, Cediranib, Palbociclib, everolimus, fluoropyrimidine, IFL, regorafenib, Reolysin, Alimta, Zykadia, Sutent, temsirolimus, axitinib, everolimus, sorafenib, Votrient, Pazopanib, IMA-901, AGS-003, cabozantinib, Vinflunine, an Hsp90 inhibitor, Ad-GM-CSF, Temazolomide, IL-2, IFN α , vinblastine, Thalomid, dacarbazine, cyclophosphamide, lenalidomide, azacytidine, lenalidomide, bortezomid, amrubicine, carfilzomib, pralatrexate, and enzastaurin.

In some embodiments, the additional therapeutic agent can comprise one or more therapeutic agents selected from the group consisting of an adjuvant, a TLR agonist, tumor necrosis factor (TNF) α , IL-1, HMGB1, an IL-10 antagonist, an IL-4 antagonist, an IL-13

antagonist, an IL-17 antagonist, an HVEM antagonist, an ICOS agonist, a treatment targeting CX3CL1, a treatment targeting CXCL9, a treatment targeting CXCL10, a treatment targeting CCL5, an LFA-1 agonist, an ICAM1 agonist, and a Selectin agonist.

In some embodiments, carboplatin, nab-paclitaxel, paclitaxel, cisplatin, pemetrexed, gemcitabine, FOLFOX, or FOLFIRI are administered to the subject. In some embodiments, the additional therapeutic agent is selected from asparaginase, busulfan, carboplatin, cisplatin, daunorubicin, doxorubicin, fluorouracil, gemcitabine, hydroxyurea, methotrexate, paclitaxel, rituximab, vinblastine, vincristine and/or combinations thereof.

COMPOSITIONS AND FORMULATIONS

The present disclosure provides compositions (including pharmaceutical and therapeutic compositions) containing the engineered cells and populations thereof, produced by the methods disclosed herein. Also provided are methods, e.g., therapeutic methods for administering the engineered cells and compositions thereof to subjects, e.g., patients.

Compositions including the engineered cells for administration, including pharmaceutical compositions and formulations, such as unit dose form compositions including the number of cells for administration in a given dose or fraction thereof are provided. The pharmaceutical compositions and formulations can include one or more optional pharmaceutically acceptable carrier or excipient. In some embodiments, the composition includes at least one additional therapeutic agent.

A pharmaceutically acceptable carrier refers to an ingredient in a pharmaceutical composition, other than an active ingredient. The pharmaceutically acceptable carrier does not interfere with the active ingredient and is nontoxic to a subject. A pharmaceutically acceptable carrier can include, but is not limited to, a buffer, excipient, stabilizer, or preservative. The pharmaceutical formulation refers to process in which different substances and/or agents are combined to produce a final medicinal product. The formulation studies involve developing a preparation of drug acceptable for patient. Additionally, a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

In some embodiments, the choice of carrier is determined in part by the particular cell

(e.g., T cell or NK cell) and/or by the method of administration. A variety of suitable formulations are available. For example, the pharmaceutical composition can contain preservatives. Suitable preservatives can include, for example, methylparaben, propylparaben, sodium benzoate, and benzalkonium chloride. In some embodiments, 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. Carriers are described, e.g., by Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980). Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG).

Suitable buffering agents include, for example, citric acid, sodium citrate, phosphoric acid, potassium phosphate, and various other acids and salts. In some embodiments, a mixture of two or more buffering agents is used. The buffering agent or mixtures thereof are typically present in an amount of about 0.001% to about 4% by weight of the total composition. Methods for preparing administrable pharmaceutical compositions are known. Exemplary methods are described in more detail in, for example, Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins; 21st ed. (May 1, 2005).

The formulations can include aqueous solutions. The formulation or composition can also contain more than one active ingredient useful for a particular indication, disease, or condition being treated with the engineered cells, preferably those with activities complementary to the cells, where the respective activities do not adversely affect one another. Such active ingredients

are suitably present in combination in amounts that are effective for the purpose intended. Thus, in some embodiments, the pharmaceutical composition can further include other pharmaceutically active agents or drugs, such as checkpoint inhibitors, fusion proteins, chemotherapeutic agents, e.g., asparaginase, busulfan, carboplatin, cisplatin, daunorubicin, doxorubicin, fluorouracil, gemcitabine, hydroxyurea, methotrexate, paclitaxel, rituximab, vinblastine, and/or vincristine.

The pharmaceutical composition in some embodiments contains the cells in amounts effective to treat or prevent the disease or condition, such as a therapeutically effective or prophylactically effective amount. Therapeutic or prophylactic efficacy in some embodiments is monitored by periodic assessment of treated subjects. The desired dosage can be delivered by a single bolus administration of the cells, by multiple bolus administrations of the cells, or by continuous infusion administration of the cells.

The cells and compositions can be administered using standard administration techniques, formulations, and/or devices. Administration of the cells can be autologous or heterologous. For example, immunoresponsive T cells or progenitors can be obtained from one subject, and administered to the same subject or a different, compatible subject after genetically modifying them in accordance with various embodiments described herein. Peripheral blood derived immunoresponsive T cells or their progeny (e.g., *in vivo*, *ex vivo* or *in vitro* derived) can be administered via localized injection, including catheter administration, systemic injection, localized injection, intravenous injection, or parenteral administration. Usually, when administering a therapeutic composition (e.g., a pharmaceutical composition containing a genetically modified immunoresponsive cell), it is generally formulated in a unit dosage injectable form (solution, suspension, emulsion).

Formulations disclosed herein include those for oral, intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual, or suppository administration. In some embodiments, the cell populations are administered parenterally. The term “parenteral,” as used herein, includes intravenous, intramuscular, subcutaneous, rectal, vaginal, and intraperitoneal administration. In some embodiments, the cells are administered to the subject using peripheral systemic delivery by intravenous, intraperitoneal, or subcutaneous injection.

The compositions in some embodiments are provided as sterile liquid preparations, e.g.,

isotonic aqueous solutions, suspensions, emulsions, dispersions, or viscous compositions, which can in some aspects be buffered to a selected pH. Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues. Liquid or viscous compositions can comprise carriers, which can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol) and suitable mixtures thereof.

Sterile injectable solutions can be prepared by incorporating the cells in a solvent, such as in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like. The compositions can contain auxiliary substances such as wetting, dispersing, or emulsifying agents (e.g., methylcellulose), pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, and/or colors, depending upon the route of administration and the preparation desired. Standard texts can in some aspects be consulted to prepare suitable preparations.

Various additives which enhance the stability and sterility of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, and sorbic acid. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

The formulations to be used for *in vivo* administration are generally sterile. Sterility can be readily accomplished, e.g., by filtration through sterile filtration membranes.

The compositions or pharmaceutical compositions as described herein can be included in a container, pack, or dispenser together with instructions for administration.

METHODS OF ADMINISTRATION

Provided are also methods of administering the cells, populations, and compositions, and uses of such cells, populations, and compositions to treat or prevent diseases, conditions, and disorders, including cancers. In some embodiments, the methods described herein can reduce the

risk of the developing diseases, conditions, and disorders as described herein.

In some embodiments, the cells, populations, and compositions, described herein are administered to a subject or patient having a particular disease or condition to be treated, e.g., via adoptive cell therapy, such as adoptive T cell therapy. In some embodiments, cells and compositions prepared by the provided methods, such as engineered compositions and end-of-production compositions following incubation and/or other processing steps, are administered to a subject, such as a subject having or at risk for the disease or condition. In some aspects, the methods thereby treat, e.g., ameliorate one or more symptom of, the disease or condition, such as by lessening tumor burden in cancer expressing an antigen recognized by the engineered T cells.

Methods for administration of cells for adoptive cell therapy are known and can be used in connection with the provided methods and compositions. For example, adoptive T cell therapy methods are described, e.g., in U.S. 2003/0170238; U.S. Pat. No. 4,690,915; Rosenberg, "Cell transfer immunotherapy for metastatic solid cancer—what clinicians need to know." *Nature reviews Clinical oncology* 8.10 (2011): 577; Themeli *et al.*, "Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy." *Nature biotechnology* 31.10 (2013): 928; Tsukahara *et al.*, "CD19 target-engineered T-cells accumulate at tumor lesions in human B-cell lymphoma xenograft mouse models." *Biochemical and biophysical research communications* 438.1 (2013): 84-89; Davila *et al.*, "CD19 CAR-targeted T cells induce long-term remission and B Cell Aplasia in an immunocompetent mouse model of B cell acute lymphoblastic leukemia." *PloS one* 8.4 (2013); each of which is incorporated herein by reference in its entirety.

In some embodiments, the cell therapy, e.g., adoptive T cell therapy, is carried out by autologous transfer, in which the T cells are isolated and/or otherwise prepared from the subject who is to receive the cell therapy, or from a sample derived from such a subject. Thus, in some aspects, the cells are derived from a subject, e.g., patient, in need of a treatment and the cells, following isolation and processing are administered to the same subject.

In some embodiments, the cell therapy, e.g., adoptive T cell therapy, is carried out by allogeneic transfer, in which the T cells are isolated and/or otherwise prepared from a subject other than a subject who is to receive or who ultimately receives the cell therapy, e.g., a first subject. In such embodiments, the cells then are administered to a different subject, e.g., a second subject, of the same species. In some embodiments, the first and second subjects are genetically

identical. In some embodiments, the first and second subjects are genetically similar. In some embodiments, the second subject expresses the same HLA class or supertype as the first subject.

In some embodiments, the subject has been treated with a therapeutic agent targeting the disease or condition, e.g. the tumor, prior to administration of the cells or composition containing the cells. In some aspects, the subject is refractory or non-responsive to the other therapeutic agent. In some embodiments, the subject has persistent or relapsed disease, e.g., following treatment with another therapeutic intervention, including chemotherapy, radiation, and/or hematopoietic stem cell transplantation (HSCT), e.g., allogenic HSCT. In some embodiments, the administration effectively treats the subject despite the subject having become resistant to another therapy.

In some embodiments, the subject is responsive to the other therapeutic agent, and treatment with the therapeutic agent reduces disease burden. In some aspects, the subject is initially responsive to the therapeutic agent, but exhibits a relapse of the disease or condition over time. In some embodiments, the subject has not relapsed. In some such embodiments, the subject is determined to be at risk for relapse, such as at high risk of relapse, and thus the cells are administered prophylactically, e.g., to reduce the likelihood of or prevent relapse. In some embodiments, the subject has not received prior treatment with another therapeutic agent.

In some embodiments, the cells are administered at a desired dosage, which in some aspects includes a desired dose or number of cells or cell type(s) and/or a desired ratio of cell types. Thus, the dosage of cells in some embodiments is based on a total number of cells (or number per kg body weight) and a desired ratio of the individual populations or sub-types, such as the CD4⁺ to CD8⁺ ratio. In some embodiments, the dosage of cells is based on a desired total number (or number per kg of body weight) of cells in the individual populations or of individual cell types. In some embodiments, the dosage is based on a combination of such features, such as a desired number of total cells, desired ratio, and desired total number of cells in the individual populations.

In some embodiments, the populations or sub-types of cells, such as CD8⁺ and CD4⁺ T cells, are administered at or within a tolerated difference of a desired dose of total cells, such as a desired dose of T cells. In some embodiments, the desired dose is a desired number of cells or a desired number of cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some embodiments, the desired dose is at or above a minimum

number of cells or minimum number of cells per unit of body weight. In some embodiments, among the total cells, administered at the desired dose, the individual populations or sub- types are present at or near a desired output ratio (such as CD4+ to CD8+ ratio), e.g., within a certain tolerated difference or error of such a ratio.

In some embodiments, the cells are administered at or within a tolerated difference of a desired dose of one or more of the individual populations or sub-types of cells, such as a desired dose of CD4+ cells and/or a desired dose of CD8+ cells. In some embodiments, the desired dose is a desired number of cells of the sub-type or population, or a desired number of such cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some embodiments, the desired dose is at or above a minimum number of cells of the population or sub-type, or minimum number of cells of the population or sub-type per unit of body weight.

Thus, in some embodiments, the dosage is based on a desired fixed dose of total cells and a desired ratio, and/or based on a desired fixed dose of one or more, e.g., each, of the individual sub-types or sub-populations. Thus, in some embodiments, the dosage is based on a desired fixed or minimum dose of T cells and a desired ratio of CD4+ to CD8+ cells, and/or is based on a desired fixed or minimum dose of CD4+ and/or CD8+ cells.

In certain embodiments, the cells or individual populations of sub-types of cells, are administered to the subject at a range of about one million to about 100 billion cells, such as, e.g., 1 million to about 50 billion cells (e.g., about 5 million cells, about 25 million cells, about 500 million cells, about 1 billion cells, about 5 billion cells, about 20 billion cells, about 30 billion cells, about 40 billion cells, or a range defined by any two of the foregoing values), such as about 10 million to about 100 billion cells (e.g., about 20 million cells, about 30 million cells, about 40 million cells, about 60 million cells, about 70 million cells, about 80 million cells, about 90 million cells, about 10 billion cells, about 25 billion cells, about 50 billion cells, about 75 billion cells, about 90 billion cells, or a range defined by any two of the foregoing values), and in some cases about 100 million cells to about 50 billion cells (e.g., about 120 million cells, about 250 million cells, about 350 million cells, about 450 million cells, about 650 million cells, about 800 million cells, about 900 million cells, about 3 billion cells, about 30 billion cells, about 45 billion cells) or any value in between these ranges.

In some embodiments, the dose of total cells and/or dose of individual sub- populations of cells is within a range of between at or about 10^4 and at or about 10^9 cells/kilograms (kg) body

weight, such as between 10^5 and 10^6 cells/kg body weight, for example, at least or at least about or at or about 1×10^5 cells/kg, 1.5×10^5 cells/kg, 2×10^5 cells/kg, or 1×10^6 cells/kg body weight. For example, in some embodiments, the cells are administered at, or within a certain range of error of, between at or about 10^4 and at or about 10^9 T cells/kilograms (kg) body weight, such as between 10^5 and 10^6 T cells/kg body weight, for example, at least or at least about or at or about 1×10^5 T cells/kg, 1.5×10^5 T cells/kg, 2×10^5 T cells/kg, or 1×10^6 T cells/kg body weight.

In some embodiments, the cells are administered at or within a certain range of error of between at or about 10^4 and at or about 10^9 CD4+ and/or CD8+ cells/kilograms (kg) body weight, such as between 10^5 and 10^6 CD4+ and/or CD8+ cells/kg body weight, for example, at least or at least about or at or about 1×10^5 CD4+ and/or CD8+ cells/kg, 1.5×10^5 CD4+ and/or CD8+ cells/kg, 2×10^5 CD4+ and/or CD8+ cells/kg, or 1×10^6 CD4+ and/or CD8+ cells/kg body weight.

In some embodiments, the cells are administered at or within a certain range of error of, greater than, and/or at least about 1×10^6 , about 2.5×10^6 , about 5×10^6 , about 7.5×10^6 , or about 9×10^6 CD4+ cells, and/or at least about 1×10^6 , about 2.5×10^6 , about 5×10^6 , about 7.5×10^6 , or about 9×10^6 CD8+ cells, and/or at least about 1×10^6 , about 2.5×10^6 , about 5×10^6 , about 7.5×10^6 , or about 9×10^6 T cells. In some embodiments, the cells are administered at or within a certain range of error of between about 10^8 and 10^{12} or between about 10^{10} and 10^{11} T cells, between about 10^8 and 10^{12} or between about 10^{10} and 10^{11} CD4+ cells, and/or between about 10^8 and 10^{12} or between about 10^{10} and 10^{11} CD8+ cells.

In some embodiments, the cells are administered at or within a tolerated range of a desired output ratio of multiple cell populations or sub-types, such as CD4+ and CD8+ cells or sub- types. In some aspects, the desired ratio can be a specific ratio or can be a range of ratios. for example, in some embodiments, the desired ratio (e.g., ratio of CD4+ to CD8+ cells) is between at or about 1:5 and at or about 5:1 (or greater than about 1:5 and less than about 5:1), or between at or about 1:3 and at or about 3:1 (or greater than about 1:3 and less than about 3:1), such as between at or about 2:1 and at or about 1:5 (or greater than about 1:5 and less than about 2:1, such as at or about 5:1, 4.5:1, 4:1, 3.5:1, 3:1, 2.5:1, 2:1, 1.9:1, 1.8:1, 1.7:1, 1.6:1, 1.5:1, 1.4:1, 1.3:1, 1.2:1, 1.1:1, 1:1, 1:1.1, 1:1.2, 1:1.3, 1:1.4, 1:1.5, 1:1.6, 1:1.7, 1:1.8, 1:1.9, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, or 1:5. In some aspects, the tolerated difference is within about 1%, about 2%, about 3%, about 4% about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about

35%, about 40%, about 45%, about 50% of the desired ratio, including any value in between these ranges. In some aspects, the TCR described here provides improved expression and activity, thereby providing therapeutic effects even at a low effector to target (E:T) ratio.

Optimal response to therapy can depend on the ability of the engineered recombinant receptors such as TCRs, to be consistently and reliably expressed on the surface of the cells and/or bind the target antigen. For example, in some cases, properties of certain recombinant receptors, e.g., TCRs, can affect the expression and/or activity of the recombinant receptor, in some cases when expressed in a cell, such as a human T cell, used in cell therapy. In some contexts, the level of expression of particular recombinant receptors, e.g., TCRs, can be low, and activity of the engineered cells, such as human T cells, expressing such recombinant receptors, may be limited due to poor expression or poor signaling activity. In some cases, consistency and/or efficiency of expression of the recombinant receptor, and activity of the receptor is limited in certain cells or certain cell populations of available therapeutic approaches. In some cases, a large number of engineered T cells (a high effector to target (E:T) ratio) is required to exhibit functional activity. In some embodiments, the desired ratio (E:T ratio) is between at or about 1:10 and at or about 10:1 (or greater than about 1:10 and less than about 10:1), or between at or about 1:1 and at or about 10:1 (or greater than about 1:1 and less than about 5:1), such as between at or about 2:1 and at or about 10:1. In some embodiments, the E:T ratio is greater than or about 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, or 10:1.

For the prevention or treatment of disease, the appropriate dosage may depend on the type of disease to be treated, the type of cells or recombinant receptors, the severity and course of the disease, whether the cells are administered for preventive or therapeutic purposes, previous therapy, the subject's clinical history and response to the cells, and the discretion of the attending physician. The compositions and cells are in some embodiments suitably administered to the subject at one time or over a series of treatments.

The cells described herein can be administered by any suitable means, for example, by bolus infusion, by injection, e.g., intravenous or subcutaneous injections, intraocular injection, periocular injection, subretinal injection, intravitreal injection, trans-septal injection, subscleral injection, intrachoroidal injection, intracameral injection, subconjunctival injection, subconjunctival injection, sub-Tenon's injection, retrobulbar injection, peribulbar injection, or posterior juxtасcleral delivery. In some embodiments, they are administered by parenteral, intrapulmonary,

and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In some embodiments, a given dose is administered by a single bolus administration of the cells. In some embodiments, it is administered by multiple bolus administrations of the cells, for example, over a period of no more than 3 days, or by continuous infusion administration of the cells.

In some embodiments, the cells are administered as part of a combination treatment, such as simultaneously with or sequentially with, in any order, another therapeutic intervention, such as an antibody or engineered cell or receptor or agent, such as a cytotoxic or therapeutic agent. The cells in some embodiments are co-administered 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 cells are administered prior to the one or more additional therapeutic agents. In some embodiments, the cells are administered after the one or more additional therapeutic agents. In some embodiments, the one or more additional agents includes a cytokine, such as IL-2, for example, to enhance persistence. In some embodiments, the methods comprise administration of a chemotherapeutic agent.

Following administration of the cells, the biological activity of the engineered cell populations in some embodiments is measured, e.g., by any of a number of known methods. Parameters to assess include specific binding of engineered T cells to the antigen, *in vivo*, e.g., by imaging, or *ex vivo*, e.g., by ELISA or flow cytometry. In certain embodiments, the ability of the engineered cells to destroy target cells can be measured using any suitable method known in the art, such as cytotoxicity assays described in, for example, Kochenderfer *et al.*, "Construction and pre-clinical evaluation of an anti-CD19 chimeric antigen receptor." *Journal of immunotherapy* (Hagerstown, Md.: 1997) 32.7 (2009): 689 and Hermans *et al.*, "The VITAL assay: a versatile fluorometric technique for assessing CTL-and NKT-mediated cytotoxicity against multiple targets *in vitro* and *in vivo*." *Journal of immunological methods* 285.1 (2004): 25-40. In certain embodiments, the biological activity of the cells is measured by assaying expression and/or secretion of one or more cytokines, such as CD107a, IFN γ , IL-2, and TNF. In some aspects the biological activity is measured by assessing clinical outcome, such as reduction

in tumor burden or load.

Repeated dosing methods are provided in which a first dose of cells is given followed by one or more second consecutive doses. The timing and size of the multiple doses of cells generally are designed to increase the efficacy and/or activity and/or function of engineered cells as described herein, when administered to a subject in adoptive therapy methods. The methods involve administering a first dose, generally followed by one or more consecutive doses, with particular time frames between the different doses.

In the context of adoptive cell therapy, administration of a given “dose” encompasses administration of the given amount or number of cells as a single composition and/or single uninterrupted administration, e.g., as a single injection or continuous infusion, and also encompasses administration of the given amount or number of cells as a split dose, provided in multiple individual compositions or infusions, over a specified period of time (e.g., no more than 3 days). Thus, in some contexts, the first or consecutive dose is a single or continuous administration of the specified number of cells, given or initiated at a single point in time. In some contexts, however, the first or consecutive dose is administered in multiple injections or infusions over a limited time period (e.g., no more than three days), such as once a day for three days or for two days or by multiple infusions over a single day period.

The cells of the first dose are administered in a single pharmaceutical composition. In some embodiments, the cells of the consecutive dose are administered in a single pharmaceutical composition.

In some embodiments, the cells of the first dose are administered in a plurality of compositions, collectively containing the cells of the first dose. In some embodiments, the cells of the consecutive dose are administered in a plurality of compositions, collectively containing the cells of the consecutive dose. In some aspects, additional consecutive doses can be administered in a plurality of compositions over a period of no more than 3 days.

With reference to a prior dose, such as a first dose, the term “consecutive dose” refers to a dose that is administered to the same subject after the prior, e.g., first, dose without any intervening doses having been administered to the subject in the interim. Nonetheless, the term does not encompass the second, third, and/or so forth, injection or infusion in a series of infusions or injections comprised within a single split dose. Thus, unless otherwise specified, a second infusion within a one, two or three-day period is not considered to be a “consecutive”

dose as used herein. Likewise, a second, third, and so-forth in the series of multiple doses within a split dose also is not considered to be an “intervening” dose in the context of the meaning of “consecutive” dose. Thus, unless otherwise specified, a dose administered a certain period of time, greater than three days, after the initiation of a first or prior dose, is considered to be a “consecutive” dose even if the subject receives a second or subsequent injection or infusion of the cells following the initiation of the first dose, so long as the second or subsequent injection or infusion occurred within the three-day period following the initiation of the first or prior dose.

Thus, unless otherwise specified, multiple administrations of the same cells over a period of up to 3 days is considered to be a single dose, and administration of cells within 3 days of an initial administration is not considered a consecutive dose and is not considered to be an intervening dose for purposes of determining whether a second dose is “consecutive” to the first.

In some embodiments, multiple consecutive doses are given, in some aspects using the same timing guidelines as those with respect to the timing between the first dose and first consecutive dose, e.g., by administering a first and multiple consecutive doses.

In some embodiments, the timing between the first dose and first consecutive dose, or a first and multiple consecutive doses, is such that each consecutive dose is given within a period of time is greater than about 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days or more. In some embodiments, the consecutive dose is given within a time period that is less than about 28 days after the administration of the first or immediately prior dose. The additional multiple additional consecutive dose or doses also are referred to as subsequent dose or subsequent consecutive dose.

The size of the first and/or one or more consecutive doses of cells are generally designed to provide improved efficacy and/or reduced risk of toxicity. In some aspects, a dosage amount or size of a first dose or any consecutive dose is any dosage or amount as described above. In some embodiments, the number of cells in the first dose or in any consecutive dose is between about 0.5×10^6 cells/kg body weight of the subject and 5×10^6 cells/kg, between about 0.75×10^6 cells/kg and 3×10^6 cells/kg or between about 1×10^6 cells/kg and 2×10^6 cells/kg.

As used herein, “first dose” is used to describe the timing of a given dose being prior to the administration of a consecutive or subsequent dose. The term does not necessarily imply that the subject has never before received a dose of cell therapy or even that the subject has

not before received a dose of the same cells or cells expressing the same recombinant receptor or targeting the same antigen.

In some embodiments, multiple doses can be administered to a subject over an extended period of time (e.g., over a period of at least 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 1 year, 2 years, 3 years, 4 years, or 5 years). A skilled medical professional may determine the length of the treatment period using any of the methods described herein for diagnosing or following the effectiveness of treatment (e.g., the observation of at least one symptom of cancer).

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLE 1. Construct design

Mouse and human IL-12 expression vectors (FIGS. 1A-1B) were designed to contain IL-12B (p40) and IL-12A (p35) separated by a peptide linker. For membrane-tethered IL-12, an immunoglobulin hinge region with either two (membrane-tethered IL-12; mt-IL-12; or smt) or three (long membrane-tethered IL-12; long mt-IL-12; or lmt) constant domains (CH2 and CH3) together with a CD4 transmembrane region were added after IL-12A. A vector expressing soluble IL-12 was also designed to contain both heavy chain variable region (VH) and light chain variable region (VL) of a tumor necrosis-targeting human IgG1, NHS-76 (NHS76-IL-12).

To generate lymphocytes expressing IL-12 in combination with a TCR or CAR, an additional nucleic acid sequence encoding TCR (FIG. 1C) or CAR (FIG. 1D) was added to the expression vectors. This additional sequence and the sequence encoding IL-12B, or NHS-76 vH, were separated by a P2A peptide sequence.

The mouse IL-12 construct is shown in FIG. 1A, and its nucleic acid sequence and corresponding amino acid sequence are SEQ ID NO: 2 and SEQ ID NO: 1, respectively. The mouse membrane-tethered IL-12 construct is shown in FIG. 1A, and its nucleic acid sequence and corresponding amino acid sequence are SEQ ID NO: 3 and SEQ ID NO: 4, respectively. The

mouse long membrane-tethered IL-12 construct is shown in FIG. 1A, and its nucleic acid sequence and corresponding amino acid sequence are SEQ ID NO: 5 and SEQ ID NO: 6, respectively. The human IL-12 construct is shown in FIG. 1B, and its nucleic acid sequence and corresponding amino acid sequence are SEQ ID NO: 7 and SEQ ID NO: 8, respectively. The human membrane-tethered IL-12 construct is shown in FIG. 1B, and its nucleic acid sequence and corresponding amino acid sequence are SEQ ID NO: 9 and SEQ ID NO: 10, respectively. The human long membrane-tethered IL-12 construct is shown in FIG. 1B, and its nucleic acid sequence and corresponding amino acid sequence are SEQ ID NO: 11 and SEQ ID NO: 12, respectively. The human NHS76-IL-12 construct is shown in FIG. 1B, and its nucleic acid sequence and corresponding amino acid sequence are SEQ ID NO: 13 and SEQ ID NO: 14, respectively.

EXAMPLE 2. Lymphocytes engineered to express IL-12 secrete IFN γ

Purified primary mouse lymphocytes were engineered to express the L202 TCR (L202) (anti-LMP2 TCR) alone, L202 plus mouse membrane-tethered IL-12 (Sample Name: mt12), or L202 plus mouse NHS-76-IL-12 (Sample Name: NHS76). Then, two days post-transduction, the indicated lymphocyte populations were co-cultured overnight with LLC-HLA-A2-Peplinker (LLW) target cells. Mouse IL-12 (mIL12) and mouse IFN γ (mIFN γ) secretion into culture media were quantified using BioLegend MAX ELISA kits for mIL12 p40 (Catalog #430707) and mIFN γ (Catalog #430801). Results in FIGS. 2A-2B indicate that both mouse IL-12 and IFN γ were expressed at higher levels in mt12 and NHS76 cells, and cytokine concentrations were further increased upon co-culture with target cells. Amino acid sequences of mouse membrane-tethered IL-12 and NHS76-IL-12 are shown in SEQ ID NO: 4 and SEQ ID NO: 14, respectively.

EXAMPLE 3. IL-12 expression increases IFN γ production in naïve lymphocytes

Naïve mouse lymphocytes isolated from lymph node were untransduced (Sample Name: UT) or transduced to express L202 TCR alone (Sample Name: L202), L202 together with membrane-tethered IL-12 (Sample Name: mt12), or L202 together with mouse NHS76-IL-12 (Sample Name: NHS76). The indicated lymphocyte populations were co-cultured overnight with Jurkat target cells. Mouse IFN γ was then measured in culture media using a mouse IFN γ ELISA MAX kit (BioLegend). As shown in FIG. 3, results indicate that the IL-12 expression stimulated

lymphocyte IFN γ production upon incubation with target cells and that IL-12 expression trans-activate naïve lymphocytes.

EXAMPLE 4: IL-12 expression on the surface of armored TCR-T in mouse lymphocytes

Primary mouse lymphocytes were isolated from lymph nodes and were transduced for 5 days with L202 TCR alone or L202 together with either mt-IL-12 or mouse NHS76-IL-12. The indicated cell types were then stained with 3 μ L of anti-mIL12 antibody PE-Cy7 to assess surface expression of IL-12. As shown in FIGS. 4A-4D, about 52% of mt-IL-12 PBMCs exhibited cell surface expression of human IL-12, whereas IL-12 was not expressed on the surface of untransduced, L202, or L202-NHS76-IL-12 cells. Amino acid sequences of human membrane-tethered IL-12 and NHS76-IL-12 are shown in SEQ ID NO: 10 and SEQ ID NO: 14, respectively.

EXAMPLE 5: Intracellular IL-12 expression of NHS-76-IL-12 mouse lymphocytes

Mouse lymphocytes were isolated from lymph nodes and then transduced for 2 days with NHS76-IL-12. As measured by staining with human Fab (FIG. 5A) or anti-IL12 antibody PE-Cy7 (FIG. 5B), lymphocytes transduced with NHS76-IL-12 exhibited intracellular IL-12 expression.

EXAMPLE 6: TCRb and human IL-12 surface staining of human PBMCs

Human PBMCs were transduced with L202 TCR alone or in combination with human mt-IL-12 or human long mt-IL-12, as indicated. 12 days post-infection, TCR and IL-12 surface expression were measured by staining with anti-TCRb antibody (TCRb-PE) and anti-IL-12 antibody (IL-12-PE-Cy7), respectively. The results in FIGS. 6A-6H indicate that cells with L202 transduction had TCR surface expression for each cell type, whereas surface IL-12 was detected on PBMCs transduced with membrane-tethered forms of IL-12. Amino acid sequences of human membrane-tethered IL-12 (mt-IL-12) and human long membrane-tethered IL-12 (human long mt-IL-12) are shown in SEQ ID NO: 10 and SEQ ID NO: 12, respectively.

EXAMPLE 7: CD69 expression in PBMCs expressing mt-IL-12 co-cultured with target cells

Untransduced human PBMCs or PBMCs engineered to express L202 TCR, L202 plus human membrane-tethered IL-12 (smt), or L202 plus human long membrane-tethered IL-12 (lmt), as indicated, were co-cultured overnight with A375-HLA-A2-peplinker (LLW) melanoma target cells on day 12 post-infection. Then, T cell activation was assessed by staining with APC anti-CD69 antibody.

FIG. 7A is a graph showing CD69 expression in human PBMCs co-cultured with A375-HLA-A2-peplinker (LLW) melanoma target cells. FIG. 7B is a graph showing CD69 expression in human PBMCs without co-culturing. As shown in FIGS. 7A-7B, PBMCs expressing IL-12 had stronger CD69 expression in comparison with L202 or untransduced T cells, when they were co-cultured with A375-HLA-A2-peplinker (LLW) melanoma target cells.

EXAMPLE 8: IFN γ expression in IL-12 armored PBMCs

Untransduced human PBMCs, or human PBMCs transduced by L202 TCR-T, L202 TCR-T plus human membrane-tethered IL-12 (smt), or L202 TCR-T plus human long membrane-tethered IL-12 (lmt) were co-cultured overnight with A375-HLA-A2-peplinker (LLW) target cells and then stained with hIFN γ -FITC to measure T cell activation. As shown in FIGS. 8A-8H, results demonstrated approximately 20% IFN γ positive rate in L202 TCR-T cells versus greater than 40% IFN γ expression in L202 TCR-T cells expressing membrane-tethered IL-12 (either smt or lmt), suggesting cell surface expression of IL-12 enhanced TCR-T cell activation.

EXAMPLE 9: CD69 expression in IL-12 armored mTCRb⁺ and mTCRb⁻ cells

Experiments were performed to determine whether the IL12 armored cells would increase activation in two types of cells: the cells with the IL12 armor, and nearby unarmored immune cells. To observe the effect of IL-12 armored cells on the unarmored cells in the vicinity, a mixed population of 50% L202 TCR transduced and 50% untransduced human PBMCs were co-cultured with A375-LLW melanoma target cells. The L202 TCR transduced human PBMCs were either transduced by L202 alone, L202 plus human membrane-tethered IL-12 (smt), or L202 plus human long membrane-tethered IL-12 (lmt). The L202 TCR has mouse TCRb.

The activation of CD3, CD8 double-positive T cells within the TCRb⁺ and TCRb⁻ populations was measured by staining for CD69. In FIG. 9A, the mTCRb⁺ population indicates cells with IL12 armor. CD69 is a marker of activation. The activation of TCRb⁺ cells was

comparable for L202 TCR-T and armored L202 TCR-T cells (FIG. 9A). In FIG. 9B, the expression of CD69 on mTCRb⁻ population indicates the activation of surrounding cells (unarmored cells). The unarmored cells were also activated. The addition of membrane-tethered IL-12 further increased the activation of PBMCs that do not express mTCRb (FIG. 9B).

EXAMPLE 10: IFN γ activation in TCRb⁺ cells was increased by the addition of membrane-tethered IL-12

Untransduced human PBMC cells, human PBMC cells that were engineered to express L202 TCR-T, L202 TCR-T plus human membrane-tethered IL-12 (mtIL12), or L202 TCR-T plus human long membrane-tethered IL-12 (long mtIL12) cells were mixed with untransduced human PBMC cells with a 1:1 ratio.

These cells were then co-cultured with A375-LLW target cells and then stained for intracellular IFN γ FITC using BD Perm/Wash buffer. Cells were gated on lymphocytes, followed by single cells, CD3⁺ cells, CD8⁺ cells and TCRb⁺ cells. As shown in FIGS. 10A-10H, data indicate that expression of membrane-tethered IL-12 enhanced T cell activation for TCR⁺ PBMCs.

EXAMPLE 11: IFN γ activation in TCRb⁻ cells is increased by the addition of membrane-tethered IL-12

Untransduced human PBMC cells, human PBMC cells that were engineered to express L202 TCR-T, L202 TCR-T plus human membrane-tethered IL-12 (mtIL12), or L202 TCR-T plus human long membrane-tethered IL-12 (long mtIL12) cells were mixed with untransduced human PBMC cells with a 1:1 ratio.

These cells were then co-cultured with A375-LLW target cells and were then stained for intracellular IFN γ FITC using BD Perm/Wash buffer. Cells were gated on lymphocytes, followed by single cells, CD3⁺ cells, CD8⁺ cells and TCRb⁻ cells. As shown in FIGS. 11A-11H, data indicate that expression of membrane-tethered IL-12 enhanced T cell activation for TCR⁻ PBMCs.

EXAMPLE 12: Phenotypic analysis of IL-12 armored TCR-T cells

On day 12 post-infection, untransduced (UT), L202 TCR-T (L202), L202 TCR-T expressing human membrane-tethered IL-12 (mt-IL12) or L202 TCR-T expressing human long membrane-tethered IL-12 (lmt-IL12) PBMCs were stained for CD4 (hCD4-Pacific Blue) and CD8 (hCD8-PerCP-Cy5.5). Results are shown in FIGS. 12A-12D. In comparison with untransduced and L202 TCR-T cells, IL-12 armored L202 TCR-T cells exhibited markedly higher CD4 expression, indicating that IL-12 enriched the CD4⁺ T cell population.

EXAMPLE 13: Flow cytometry staining to quantify central memory and effector memory T cells

Untransduced CD4⁺ PBMCs in the experiment above were stained for the effector memory marker CD45RO (hCD45RO-FITC antibody) and the central memory marker CCR7 (CCR7-R-PE antibody). As shown in FIG. 13, flow cytometry analysis revealed that approximately 41% of PBMCs were effector memory cells whereas 58% were central memory cells.

EXAMPLE 14: Memory phenotype of IL-12 armored CD4⁺ TCR-T cells

CD4⁺ untransduced (UT), L202 TCR-T (L202), L202 TCR-T expressing human membrane-tethered IL-12 (mt-IL12) or L202 TCR-T expressing human long membrane-tethered IL-12 (lmt-IL12) PBMCs were stained for CD45RO and CCR7. As shown in FIGS. 14A-14D, results indicate that the expression of surface-bound IL-12 shifted CD4⁺ PBMCs toward an effector memory phenotype.

EXAMPLE 15: Memory phenotype of IL-12 armored CD8⁺ TCR-T cells

CD8⁺ untransduced (UT), L202 TCR-T (L202), L202 TCR-T expressing human membrane-tethered IL-12 (mt-IL12) or L202 TCR-T expressing human long membrane-tethered IL-12 (lmt-IL12) PBMCs were stained for CD45RO and CCR7. As shown in FIGS. 15A-15D, results indicate that the expression of surface-bound IL-12 shifted CD8⁺ PBMCs toward an effector memory phenotype.

EXAMPLE 16: IL-12 is not released from cells expressing membrane-tethered IL-12

Jurkat cells were either untransduced (UT), or transduced to express IL-12, NHS76-IL-12, or membrane-tethered IL-12. On day 5 post transduction, 1×10^6 cells were plated in 1 ml of cell culture medium in a 12-well plate. After 24 hours, the cell cultures were centrifuged and supernatants were collected. Concentrations of IL-12 in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA). As shown in FIG. 16, Jurkat cells expressing membrane-tethered IL-12 had the lowest level of IL-12 in the cell culture medium. In contrast, Jurkat cells expressing IL-12 or NHS76-IL-12 had a high extracellular IL-12 level (e.g., 3000-5000 pg/mL) in the medium. The results suggest that the membrane-tethered IL-12 with CH2 and CH3 domains and a CD4 transmembrane region can be securely tethered to the cell membrane and will not be released into the cell culture medium.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A cell expressing
 - (a) an exogenous T cell receptor (TCR), or a chimeric antigen receptor (CAR); and
 - (b) IL-12.
2. The cell of claim 1, wherein the IL-12 is a membrane tethered IL-12.
3. The cell of claim 2, wherein the membrane tethered IL-12 comprises a CD4 transmembrane region.
4. The cell of claim 3, wherein the membrane tethered IL-12 comprises an immunoglobulin CH2 domain, an immunoglobulin CH3 domain, and a CD4 transmembrane region.
5. The cell of claim 3, wherein the membrane tethered IL-12 comprises two or more immunoglobulin CH2 domains, one or more immunoglobulin CH3 domains, and a CD4 transmembrane region.
6. The cell of claim 4 or 5, wherein the immunoglobulin CH2 domain is a wild-type immunoglobulin constant domain.
7. The cell of claim 4 or 5, wherein the amino acid at position 235 (EU numbering) of the immunoglobulin CH2 domain is Glu and the amino acid residue at position 297 (EU numbering) of the immunoglobulin CH2 domain is Gln.
8. The cell of any one of claims 2-7, wherein the membrane tethered IL-12 further comprises an immunoglobulin hinge region.
9. The cell of any one of claims 4-8, wherein the immunoglobulin CH2 domain is a human immunoglobulin CH2 domain, the immunoglobulin CH3 domain is a human immunoglobulin CH3 domain, and the CD4 transmembrane region is a human CD4

transmembrane region.

10. The cell of claim 1, wherein the IL-12 is a soluble IL-12.
11. The cell of any one of claims 1-10, wherein the IL-12 is linked to a tumor-targeting antibody or antigen-binding fragment thereof.
12. The cell of claim 11, wherein the tumor-targeting antibody or antigen binding fragment thereof is a single-chain variable fragment (scFv).
13. The cell of claim 11 or 12, wherein the tumor-targeting antibody is NHS76.
14. The cell of any one of claims 1-13, wherein the IL-12 is a human IL-12 or a mouse IL-12.
15. The cell of any one of claims 1-14, wherein the TCR or CAR targets BCMA, CD19, CD22, CD30, CD33, CD56, CD123 (IL-3R), CEA, IL13Ra2, ALPP, EBV-related antigens (e.g., LMP2), EGFR, EGFRvIII, GD2, GPC3, HER2, a HPV-related antigen (e.g., E6 or E7), MAGE (e.g., MAGE-A3), Mesothelin, MUC-1, NY-ESO-1, PSCA, PSMA, ROR1, WT1, or Claudin 18.2.
16. The cell of any one of claims 1-15, wherein the CAR comprises an extracellular domain, wherein the extracellular domain is a single chain variable fragment (scFv), a ligand (e.g., a receptor-binding ligand), or an antibody mimetic.
17. A vector comprising:
 - a) a first nucleic acid sequence encoding an IL-12 alpha subunit and an IL-12 beta subunit;
 - b) a second nucleic acid sequence encoding one or more immunoglobulin CH2 domains, one or more immunoglobulin CH3 domains, and a transmembrane region, wherein the first nucleic acid sequence and the second nucleic acid sequence are linked by a first linker sequence.

18. The vector of claim 17, wherein the first linker sequence encodes an immunoglobulin hinge polypeptide sequence.
19. The vector of claim 17 or 18, wherein the transmembrane region is a CD4 transmembrane region.
20. The vector of claim 17 or 18, wherein the transmembrane region is an immunoglobulin transmembrane region.
21. The vector of claim 17-20, wherein the IL-12 alpha subunit and the IL-12 beta subunit are linked by a linker peptide sequence.
22. The vector of any one of claims 17-21, wherein the vector further comprises a sequence encoding a signal peptide.
23. The vector of any one of claims 17-22, wherein the IL-12 alpha subunit is a human IL-12 alpha subunit, the IL-12 beta subunit is a human IL-12 beta subunit, the immunoglobulin CH2 domain is a human immunoglobulin CH2 domain, the immunoglobulin CH3 domain is a human immunoglobulin CH3 domain.
24. The vector of any one of claims 17-23, wherein the vector further comprises a third nucleic acid sequence encoding a T cell receptor (TCR), or a chimeric antigen receptor (CAR).
25. The vector of claim 24, wherein the third nucleic acid sequence is linked to the first nucleic acid by a second linker sequence.
26. The vector of claim 25, wherein the second linker sequence encodes a P2A sequence.
27. The vector of any one of claims 17-26, wherein the first nucleic acid and the second nucleic acid are under control of a regulatory element (e.g., a promotor).

28. The vector of any one of claims 24-26, wherein the first nucleic acid, the second nucleic acid, and the third nucleic acid are under control of a regulatory element (e.g., a promotor).
29. A vector comprising:
- a) a first nucleic acid sequence encoding a heavy chain variable region (VH) and a light chain variable region (VL) of a tumor-targeting antibody;
 - b) a second nucleic acid sequence encoding an IL-12 alpha subunit and an IL-12 beta subunit,
- wherein the first nucleic acid sequence and the second nucleic acid sequence are linked by a first linker sequence.
30. The method of claim 29, wherein the tumor-targeting antibody targets a tumor-associated antigen.
31. The method of claim 30, wherein the tumor-targeting antibody is NHS76.
32. The vector of any one of claims 29-31, wherein the vector further comprises a nucleic acid encoding a signal peptide.
33. The vector of claim 32, wherein the signal peptide is a human signal peptide, the IL-12 alpha subunit is a human IL-12 alpha subunit, and the IL-12 beta subunit is a human IL-12 beta subunit.
34. The vector of any one of claims 29-33, wherein the vector further comprises a third nucleic acid sequence encoding a T cell receptor (TCR), or a chimeric antigen receptor (CAR).
35. The vector of claim 34, wherein the third nucleic acid sequence is linked to 5' of the first nucleic acid by a second linker sequence.
36. The vector of claim 35, wherein the second linker sequence encodes a P2A sequence.

37. The vector of any one of claims 29-36, wherein the first nucleic acid and the second nucleic acid are under control of a regulatory element (e.g., a promotor).
38. The vector of any one of claims 34-36, wherein the first nucleic acid, the second nucleic acid, and the third nucleic acid are under control of a regulatory element (e.g., a promotor).
39. A vector comprising, in a 5' to 3' direction
- a) a first nucleic acid sequence encoding an exogenous T cell receptor (TCR), or a chimeric antigen receptor (CAR);
 - b) a second nucleic acid sequence encoding a signal peptide, an IL-12 alpha subunit and an IL-12 beta subunit;
- wherein the first nucleic acid sequence and the second nucleic acid sequence are linked by a linker sequence.
40. The vector of claim 39, wherein the IL-12 alpha subunit and the IL-12 beta subunit are linked by a linker peptide sequence.
41. The vector of claim 39 or 40, wherein the linker sequence encodes a P2A sequence
42. A fusion polypeptide, comprising
- a) a first region comprising an IL-12 alpha subunit, and an IL-12 beta subunit, wherein the IL-12 alpha subunit and the IL-12 beta subunit are linked by a linker peptide sequence,
 - b) a second region comprising one or more immunoglobulin CH2 domains, one or more immunoglobulin CH3 domains, and a transmembrane region.
43. The polypeptide of claim 42, wherein the first region and the second region are linked by an immunoglobulin hinge peptide.
44. A fusion polypeptide, comprising
- a) a first region comprising an IL-12 alpha subunit, and an IL-12 beta subunit, wherein the IL-12 alpha subunit and the IL-12 beta subunit are linked by a first linker peptide

sequence,

- b) a second region comprising a heavy chain variable region (VH) and a light chain variable region (VL) of a tumor-targeting antibody, wherein the VH and the VL are linked by a second linker peptide sequence,

wherein the first region and the second region are linked by a third linker peptide sequence.

45. The polypeptide of claim 44, wherein the third linker polypeptide sequence has a sequence that is at least 80% identical to SEQ ID NO: 17.

46. A nucleic acid encoding the polypeptide in any one of claims 42-45.

47. A vector comprising the nucleic acid in claim 46.

48. A cell expressing the fusion polypeptide in any one of claims 42-45.

49. A cell comprising the vector of any one of claims 17-41 and 47.

50. The cell of claim 49, wherein the cell secretes a higher level of a cytokine as compared to a same cell except that the cell does not comprise the vector.

51. The cell of claim 49, wherein the cell stimulates one or more cells in the vicinity of the cell to secrete a cytokine.

52. The cell of claim 50 or 51, wherein the cytokine is an IFN γ .

53. The cell of claim 49, wherein the cell expresses a higher level of an early TCR activation marker as compared to a same cell except that the cell does not comprise the vector, and/or stimulates one or more cells in the vicinity of the cell to express the early TCR activation marker.

54. The cell of claim 53, wherein the activation maker is a CD69.

55. The cell of any one of claims 1-16 and 48-54, wherein the cell is a cell line.
56. The cell of any one of claims 1-16 and 48-54, wherein the cell is a primary cell obtained from a subject (e.g., a human subject).
57. The cell of any of claims 1-16 and 48-54, wherein the cell is an immune cell (e.g., a lymphocyte).
58. The cell of any of claims 1-16 and 48-54, wherein the cell is a tumor-infiltrating lymphocyte (TIL) or a NK cell (e.g., a CAR-NK cell).
59. The cell of any of claims 1-16 and 48-54, wherein the cell is a T cell.
60. The cell of claim 59, wherein the T-cell is isolated from a human subject.
61. The cell of claim 59 or 60, wherein the T cell is CD8+.
62. The cell of any one of claims 59-61, wherein the T cell is CD4+.
63. The vector of any one of claims 17-41 and 47, wherein the vector is an expression vector, a viral vector, a retroviral vector, or a lentiviral vector.
64. The vector of claim 63, wherein the retroviral vector is pMP71.
65. A method for producing a cell, comprising introducing the vector of any one of claims 17-41, 47, 63 and 64 into the cell *in vitro* or *ex vivo*.
66. The method of claim 65, wherein the vector is introduced into the cell by transduction.
67. A method of treating a subject having a cancer, the method comprising administering to the subject in need thereof, an effective amount of the cell in any one of claims 1-16 and 48-62.

68. The method of claim 67, wherein the cancer is a heterogeneous cancer.
69. The method of claim 67, wherein the cancer is a homogeneous cancer.
70. The method of any one of claims 67-69, wherein the cell is isolated from peripheral blood mononuclear cells (PBMCs) of the subject.
71. A method of treating a subject having a cancer, the method comprising administering to the subject in need thereof,
- a) an effective amount of cells expressing a T cell receptor (TCR), or a chimeric antigen receptor (CAR); and
 - b) an effective amount of a protein comprising an IL-12 and a tumor-targeting antibody or antigen binding fragment thereof.
72. The method of claim 71, wherein the cell is isolated from peripheral blood mononuclear cells of the subject.
73. A method of treating a human subject having a cancer, the method comprising providing cells collected from the human subject or a different human subject; introducing the vector of any one of claims 17-41 and 47 in to the cells; culturing and expanding the cells; and administering an effective amount of composition comprising the cells to the subject.
74. The method of claim 73, wherein the cancer is a heterogeneous cancer.
75. The method of claim 73, wherein the cancer is a homogeneous cancer.
76. The method of any one of claims 73-75, wherein the cells are peripheral blood mononuclear cells (PBMC).
77. The method of any one of claims 73-75, wherein the cells are tumor-infiltrating lymphocytes and the vector comprises a nucleic acid encoding IL-12.

78. The method of claim 77, wherein the IL-12 is a membrane tethered IL-12.
79. The method of any one of claims 73-75, wherein the cells are T cells and the vector comprises a nucleic acid encoding TCR or CAR.
80. The method of claim 79, wherein the vector further comprises a nucleic acid encoding IL-12.
81. The method of claim 80, wherein the IL-12 is a membrane tethered IL-12.

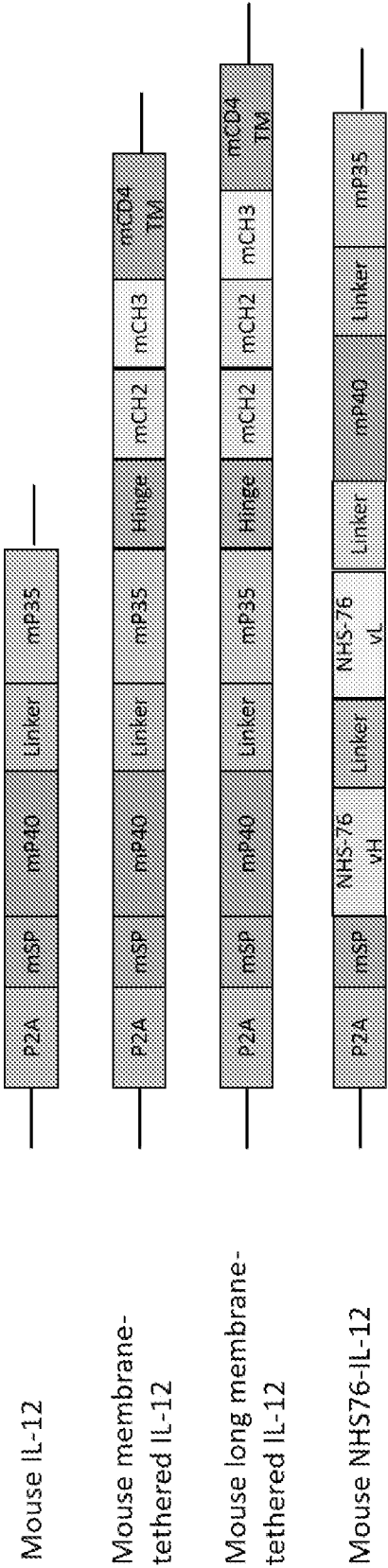


FIG. 1A

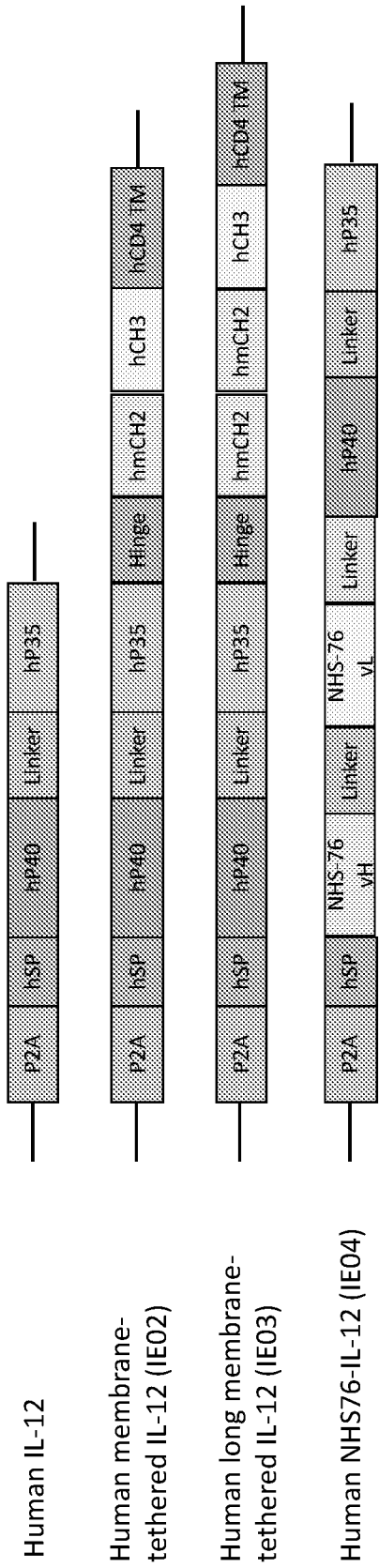


FIG. 1B

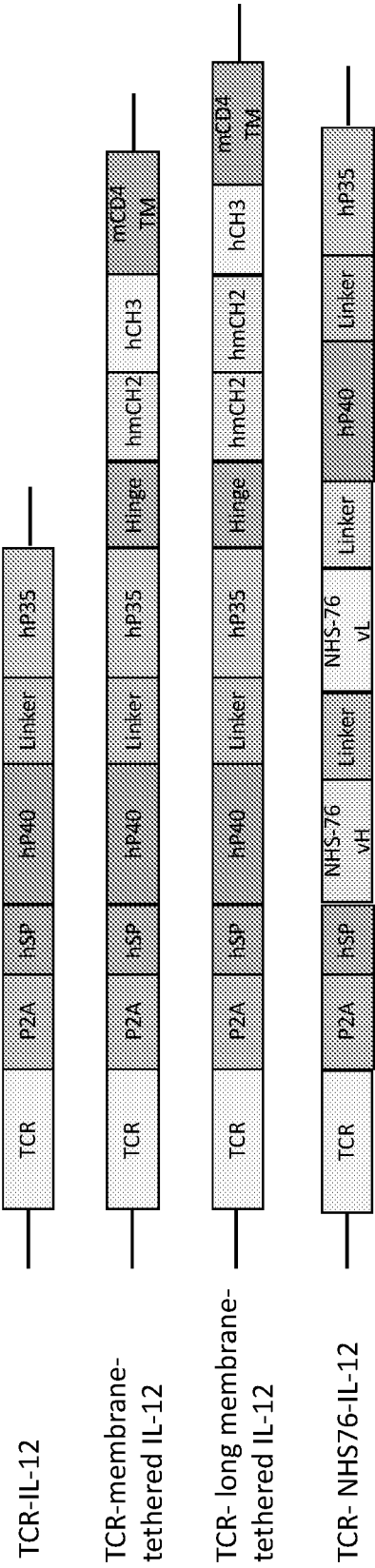


FIG. 1C

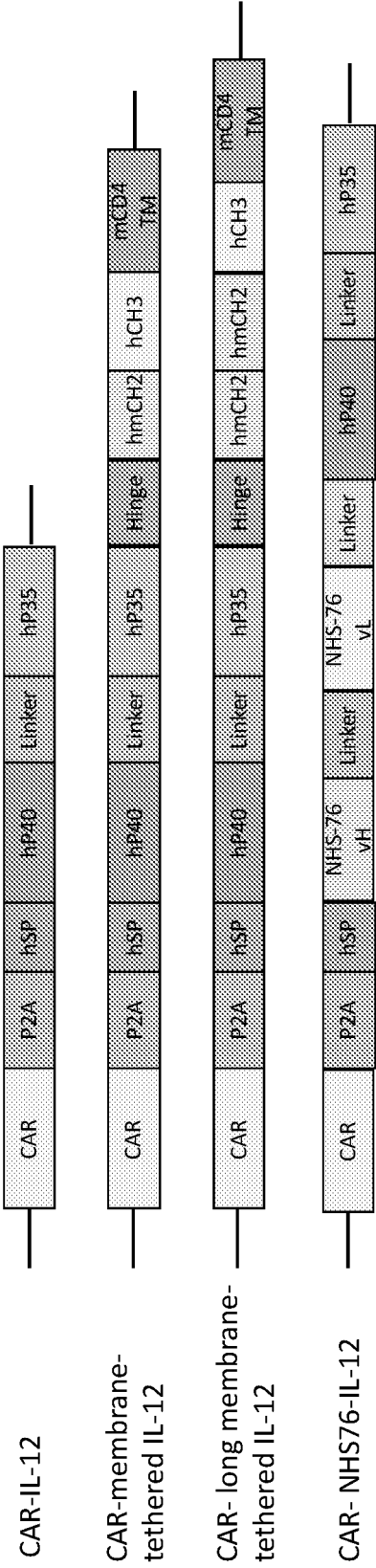


FIG. 1D

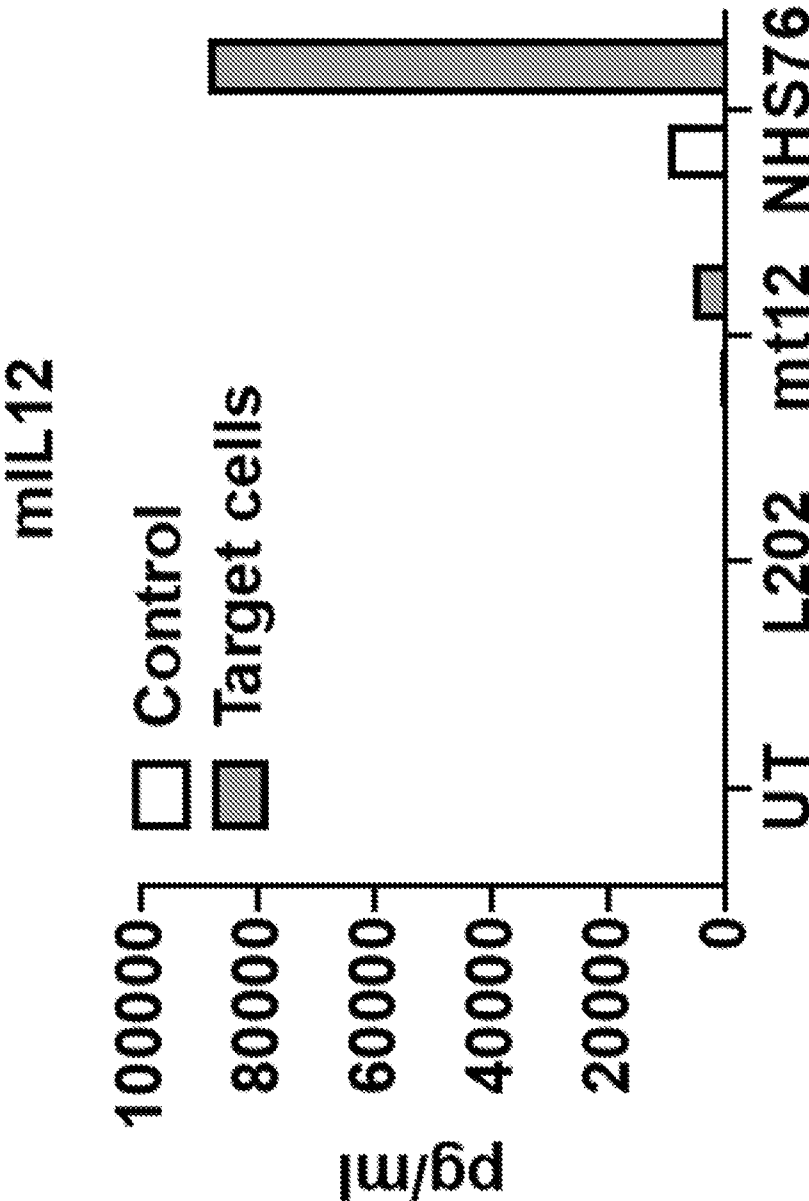


FIG. 2A

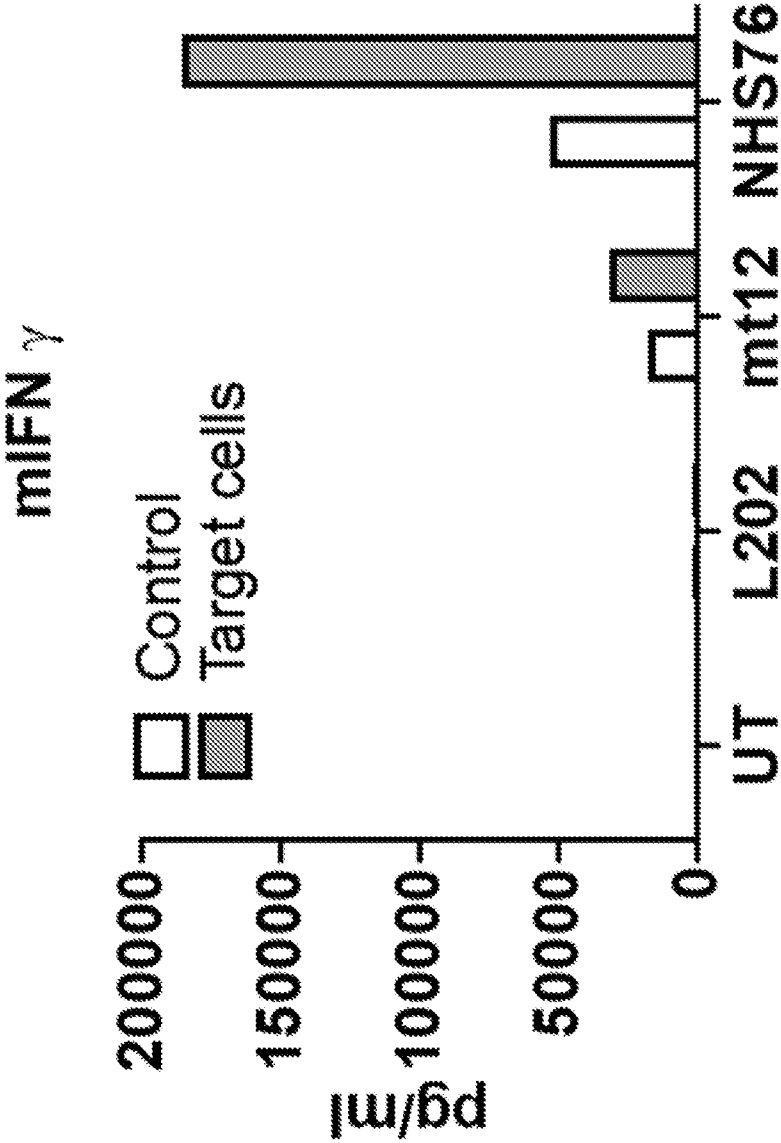


FIG. 2B

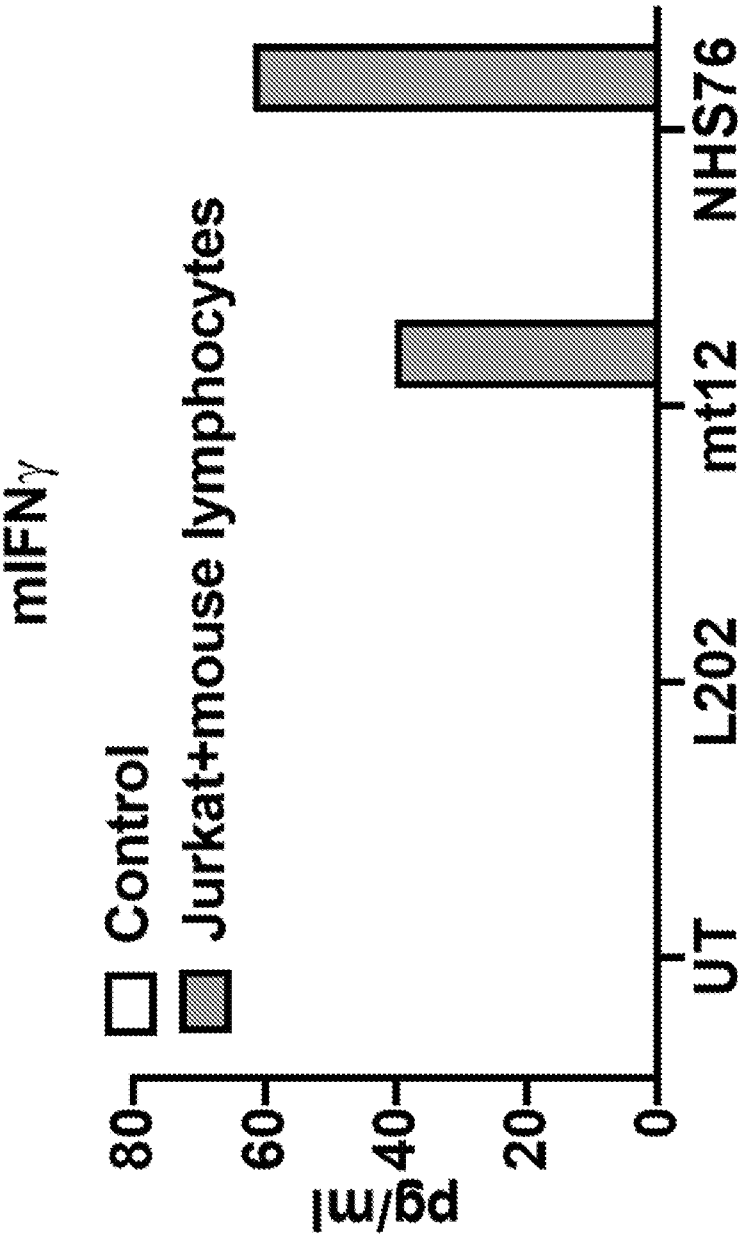
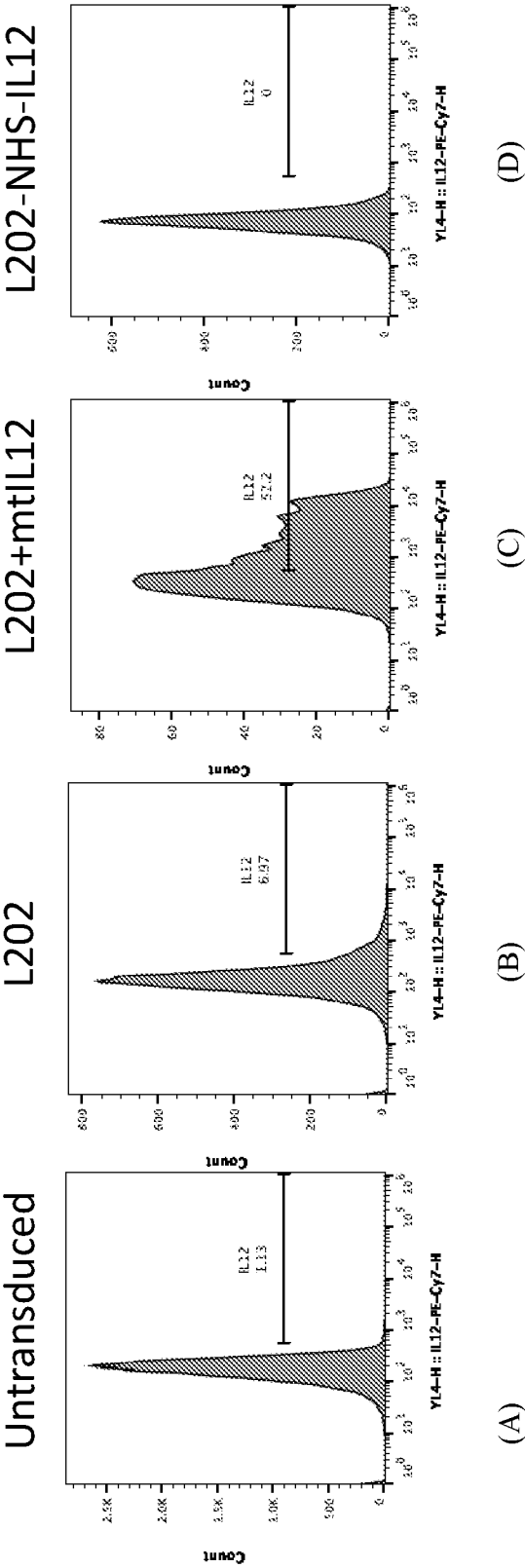


FIG. 3



FIGS. 4A-4D

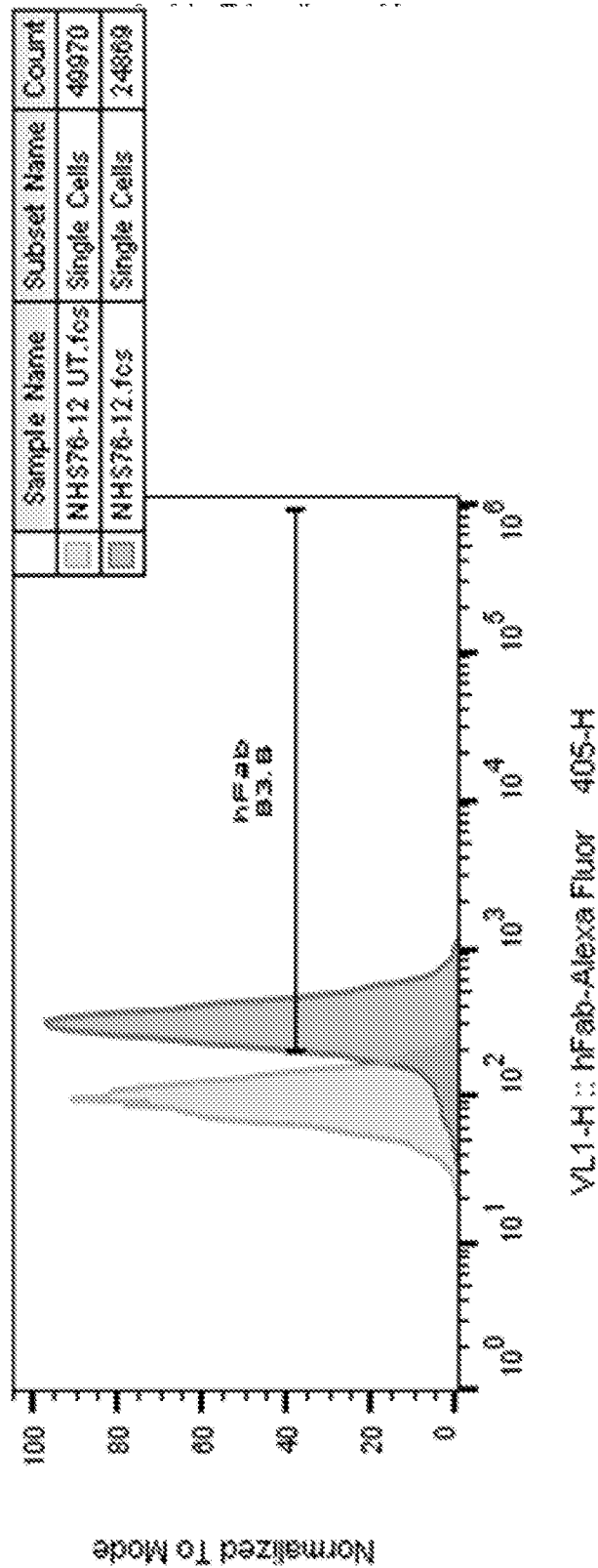


FIG. 5A

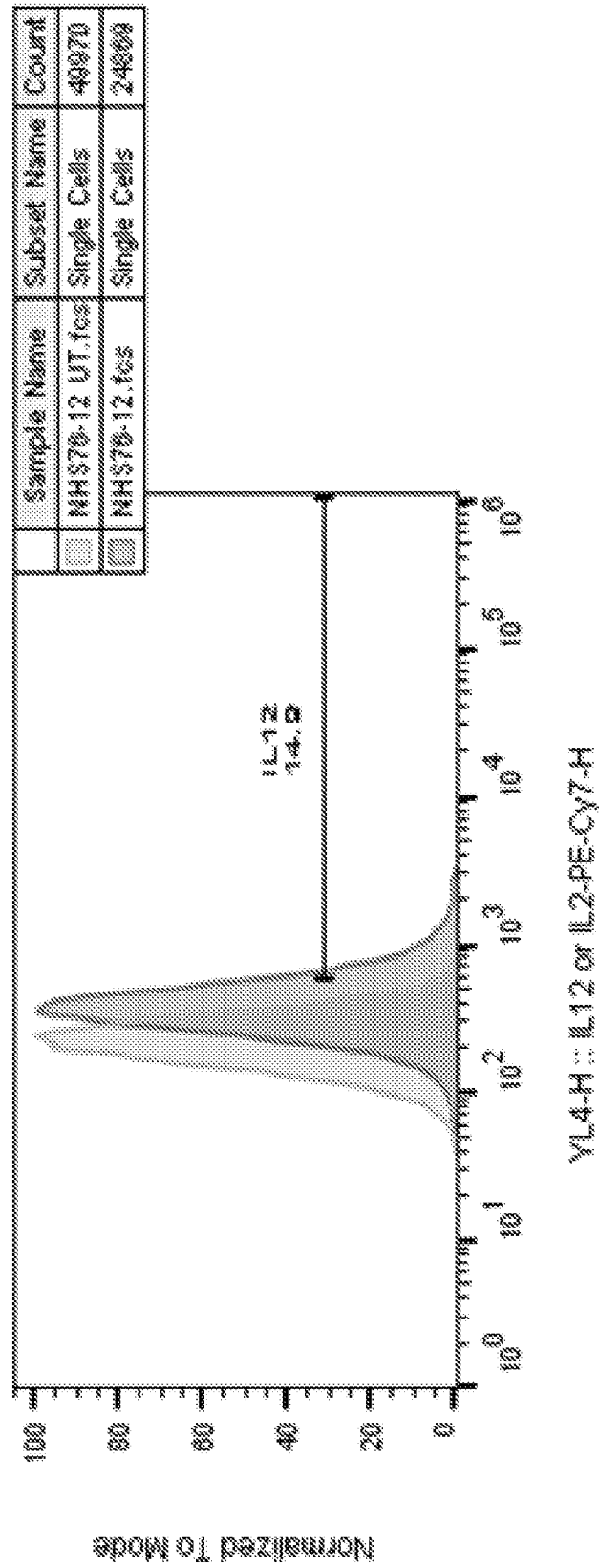
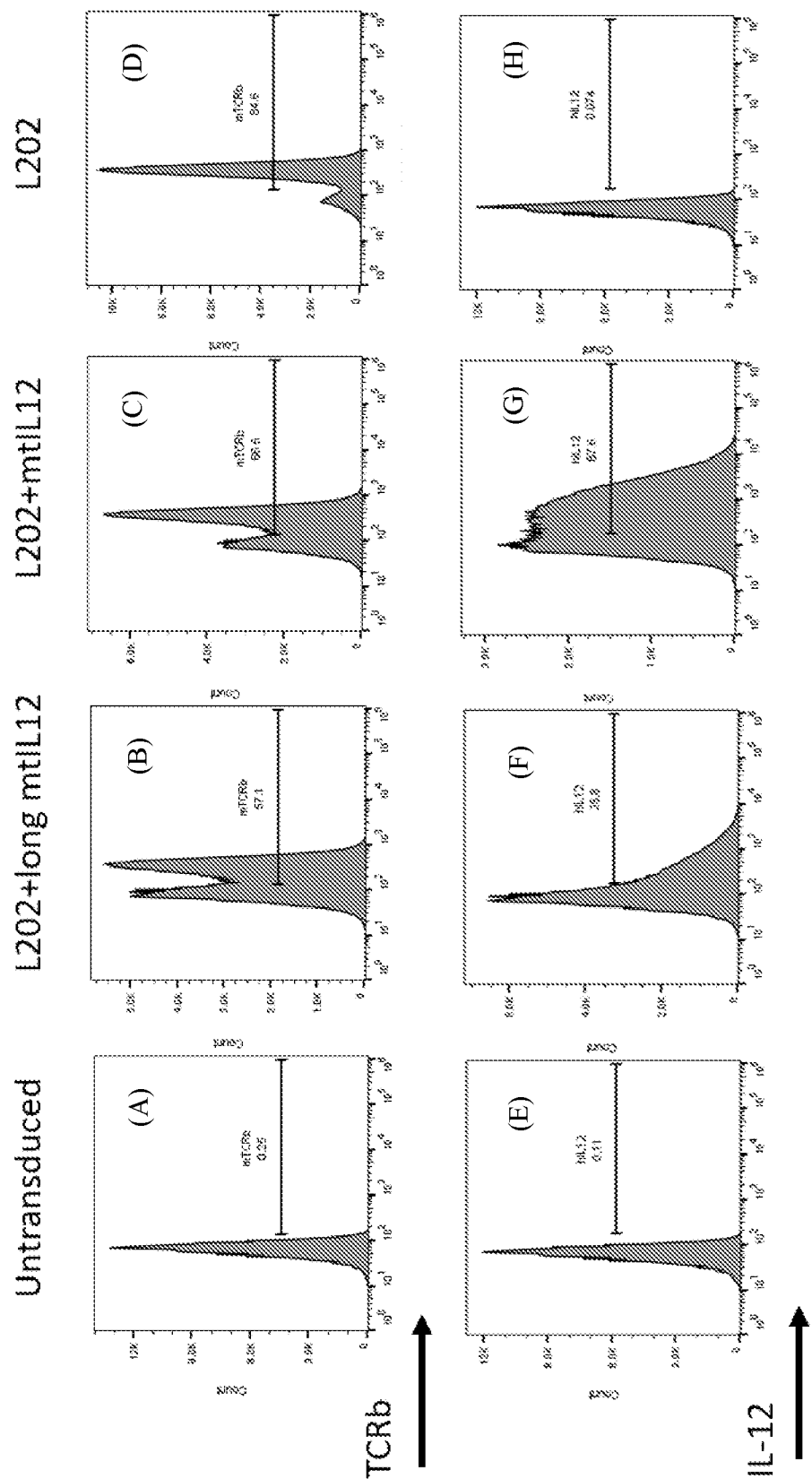
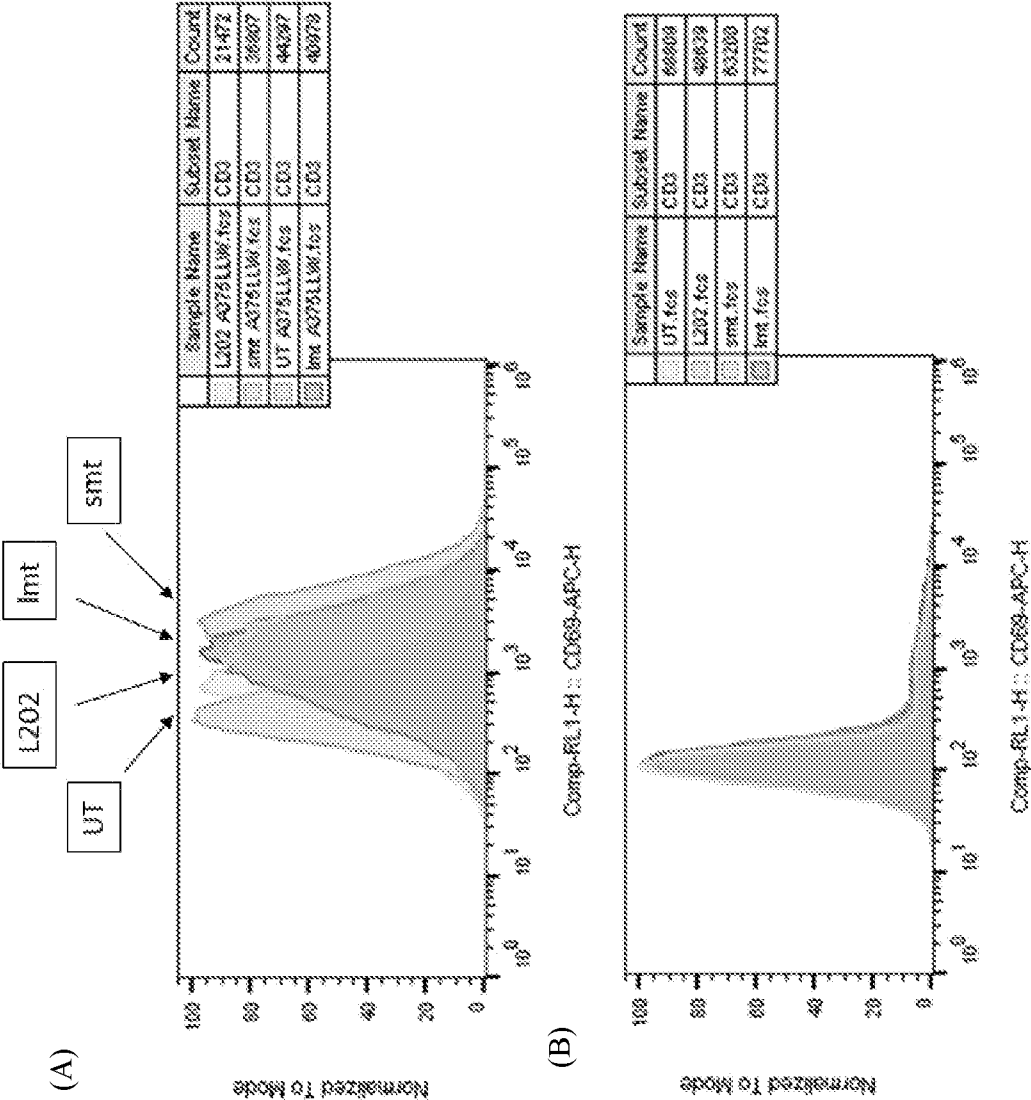


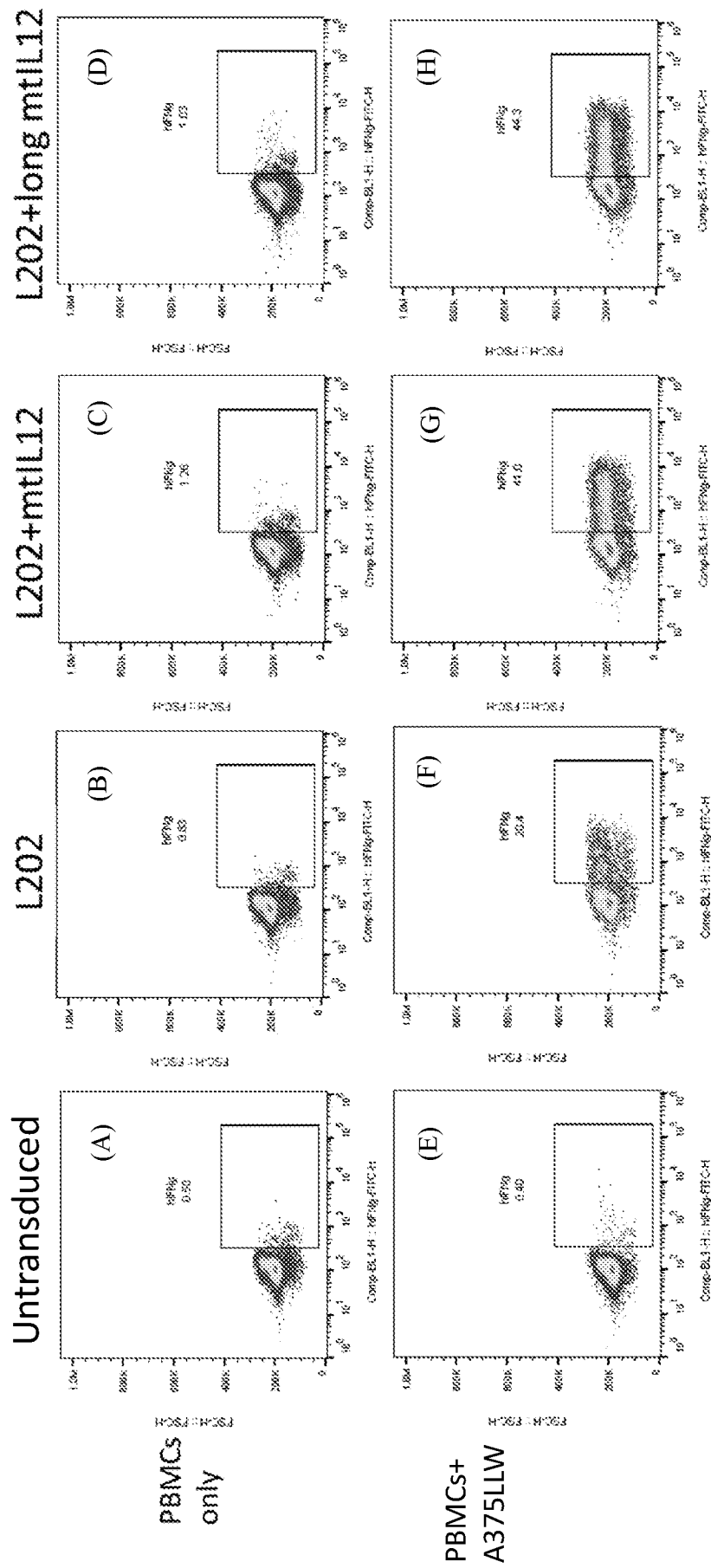
FIG. 5B



FIGS. 6A-6H



FIGS. 7A-7B



FIGS. 8A-8H

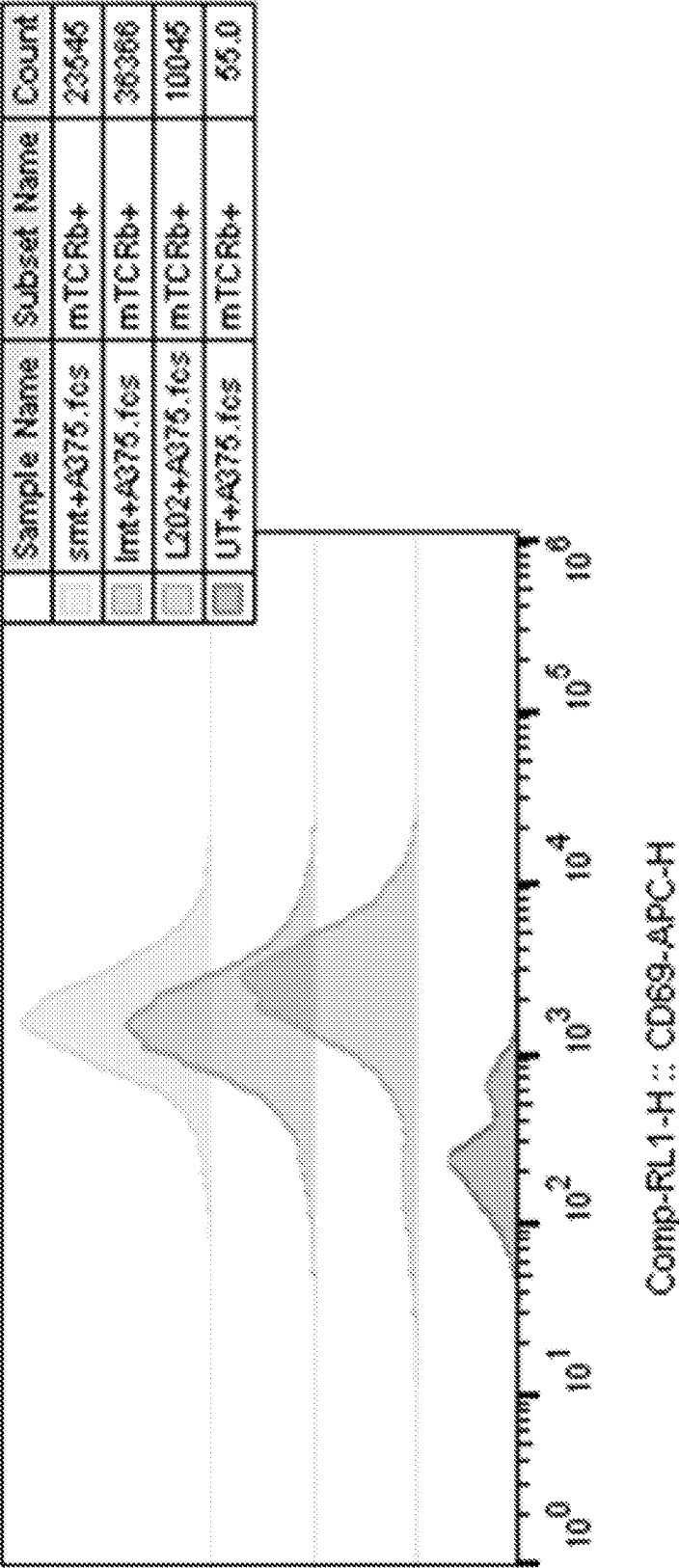


FIG. 9A

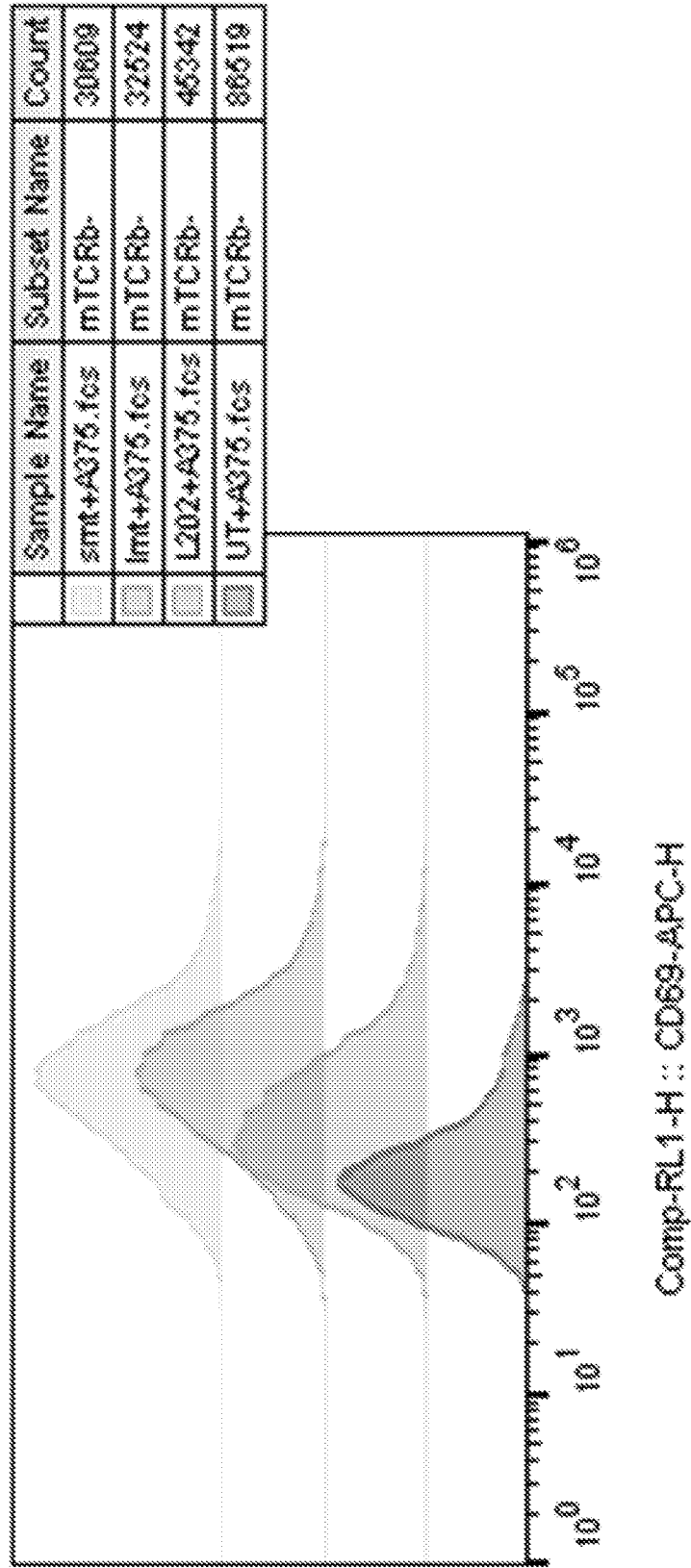
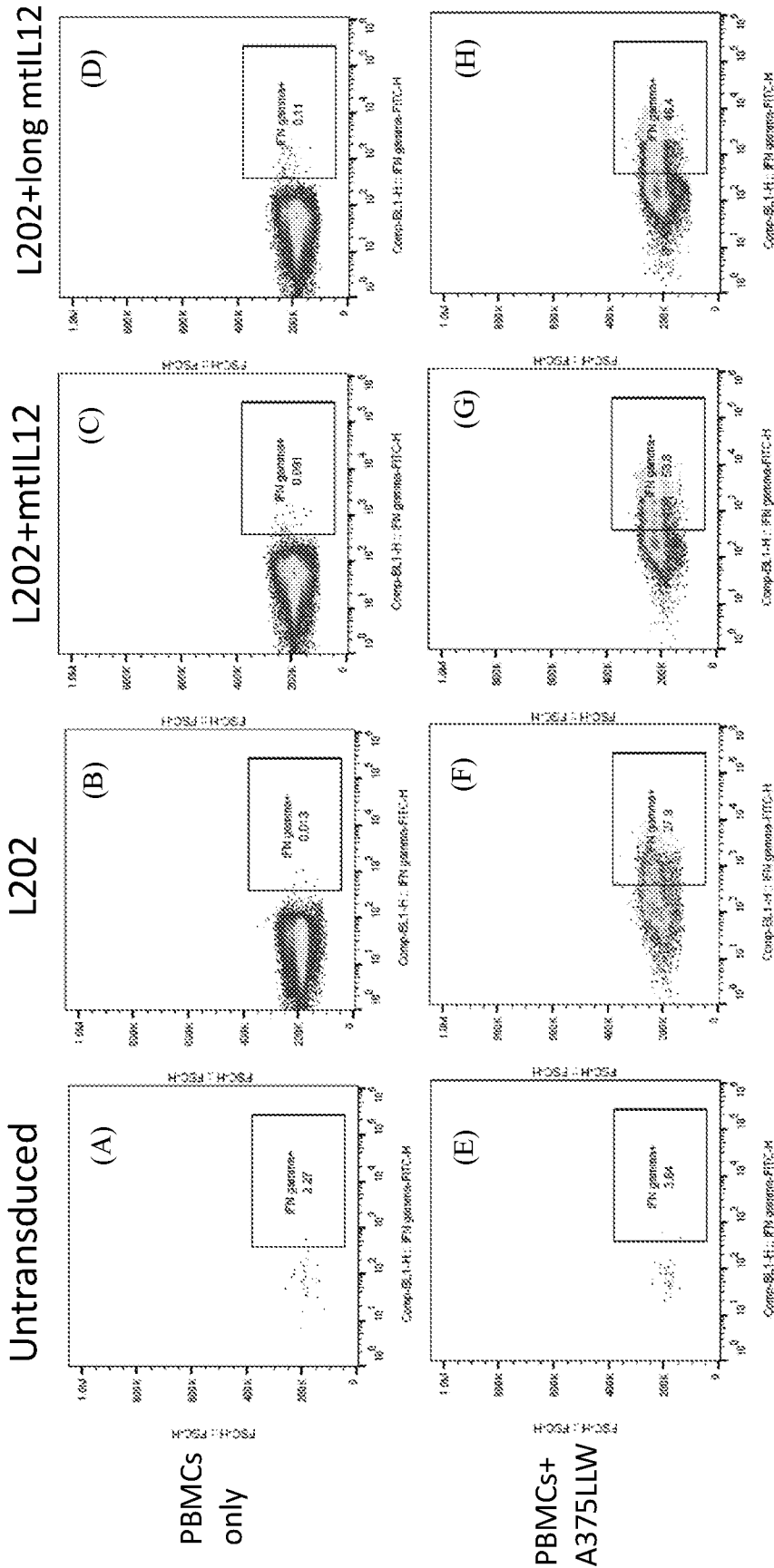
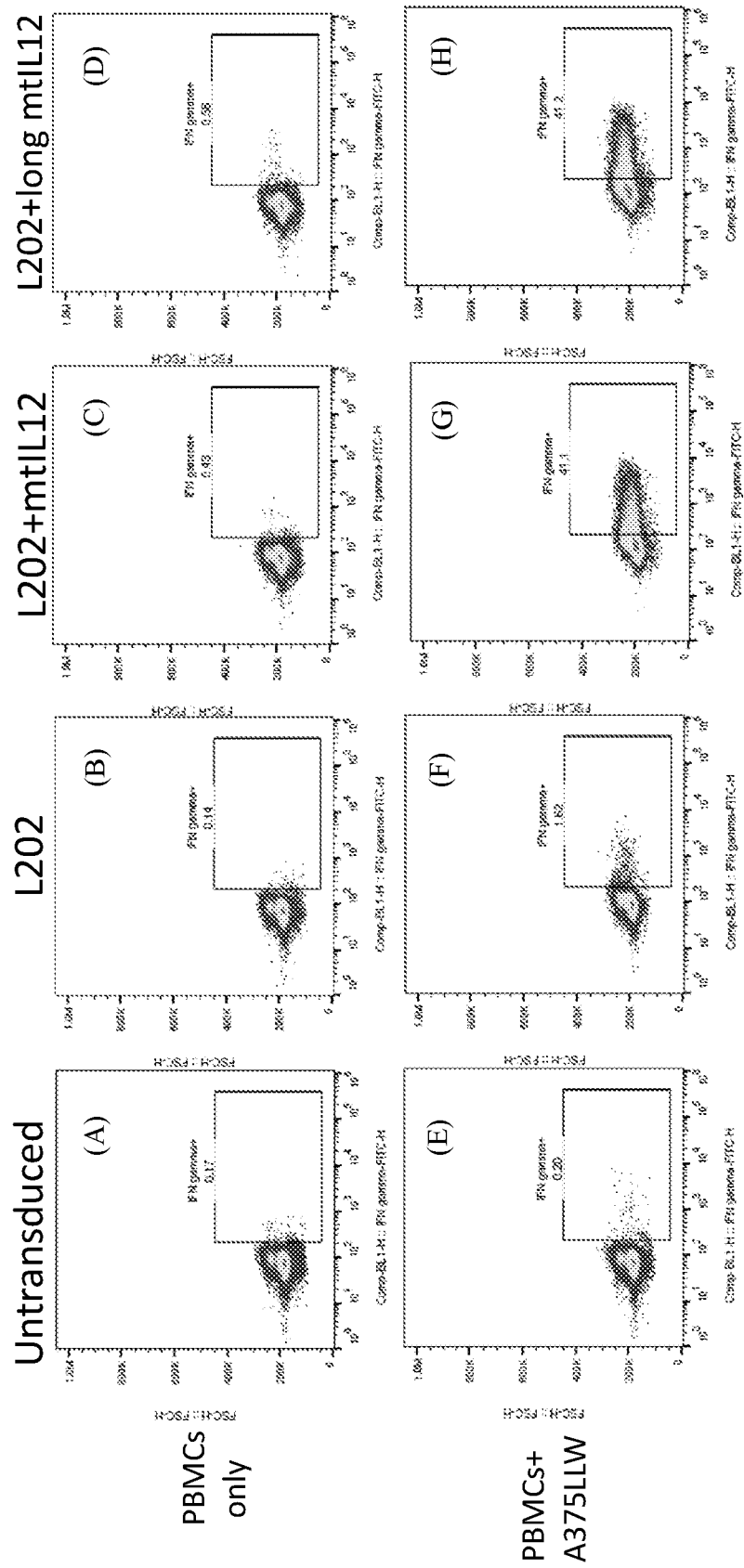


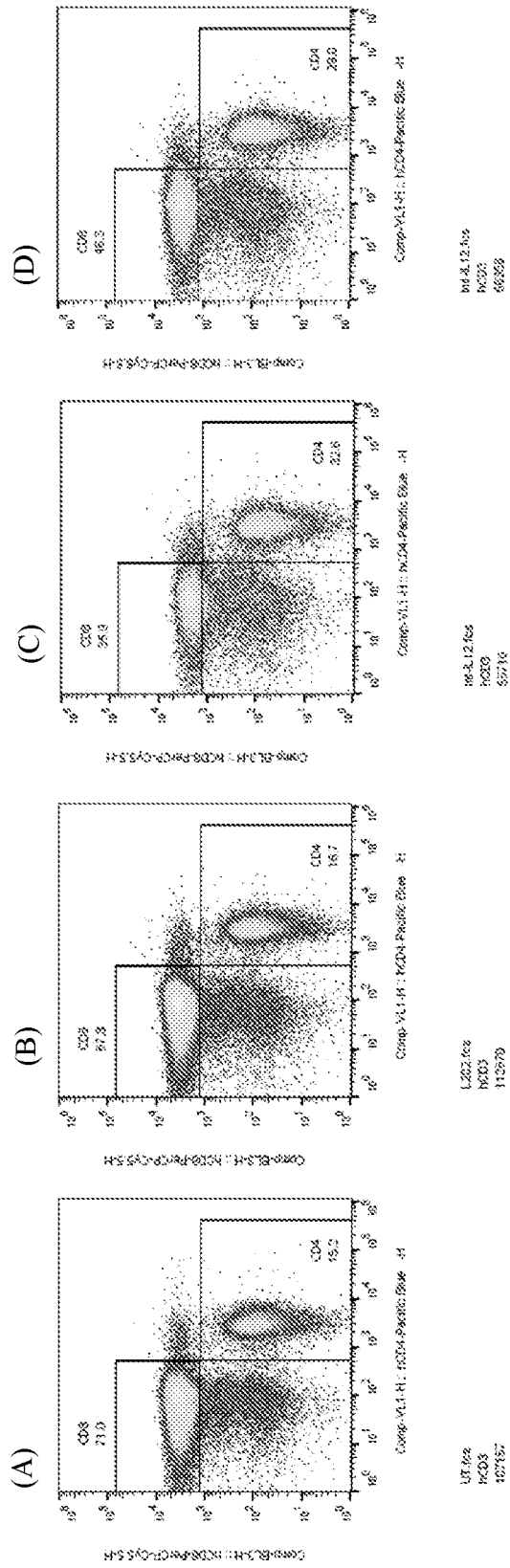
FIG. 9B



FIGS. 10A-10H



FIGS. 11A-11H



FIGS. 12A-12D

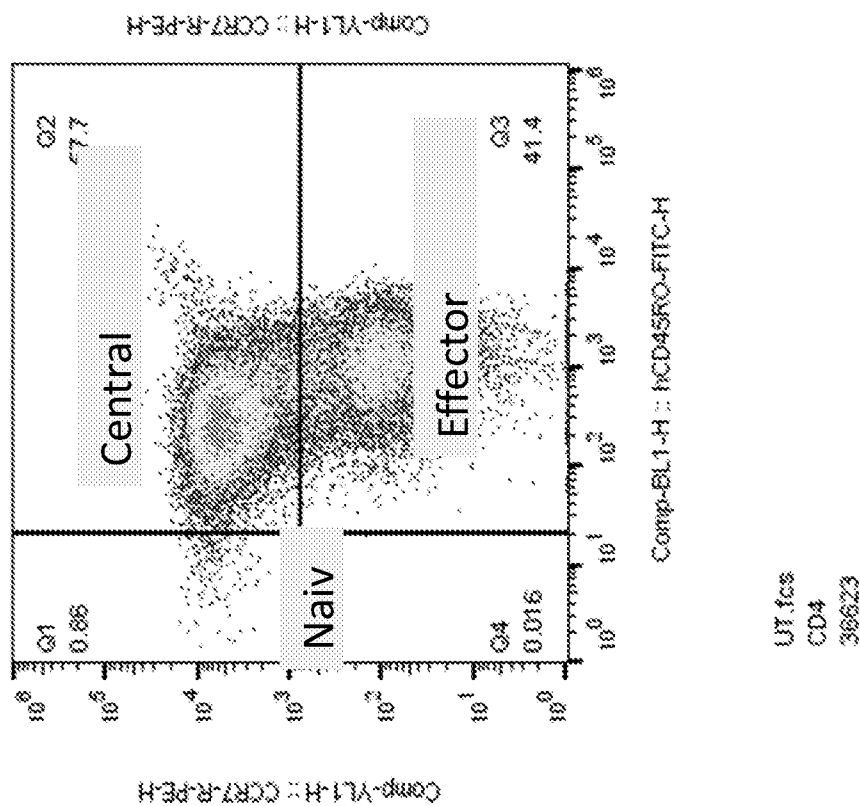
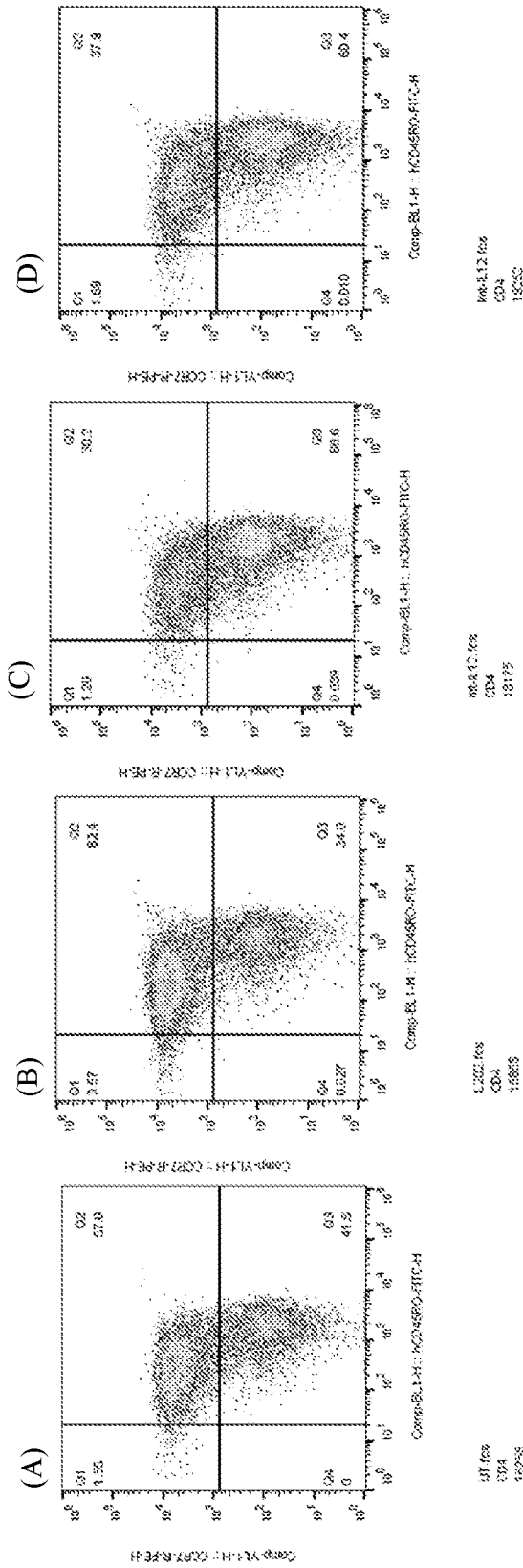
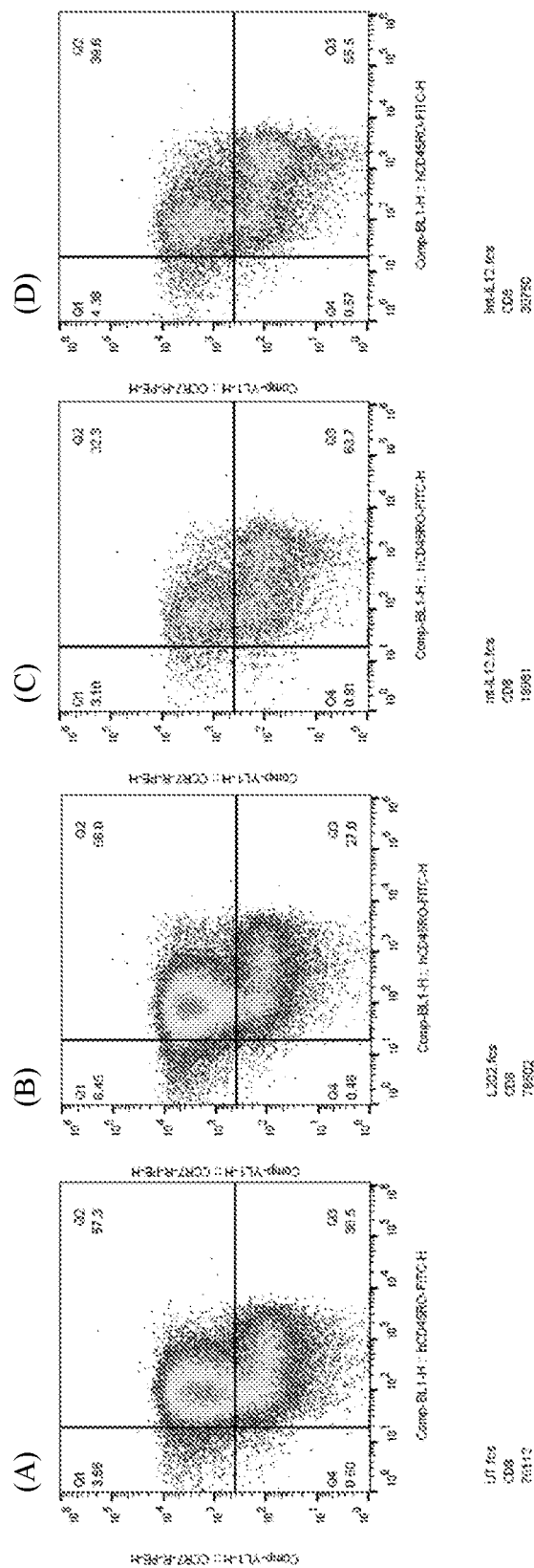


FIG. 13



FIGS. 14A-14D



FIGS. 15A-15D

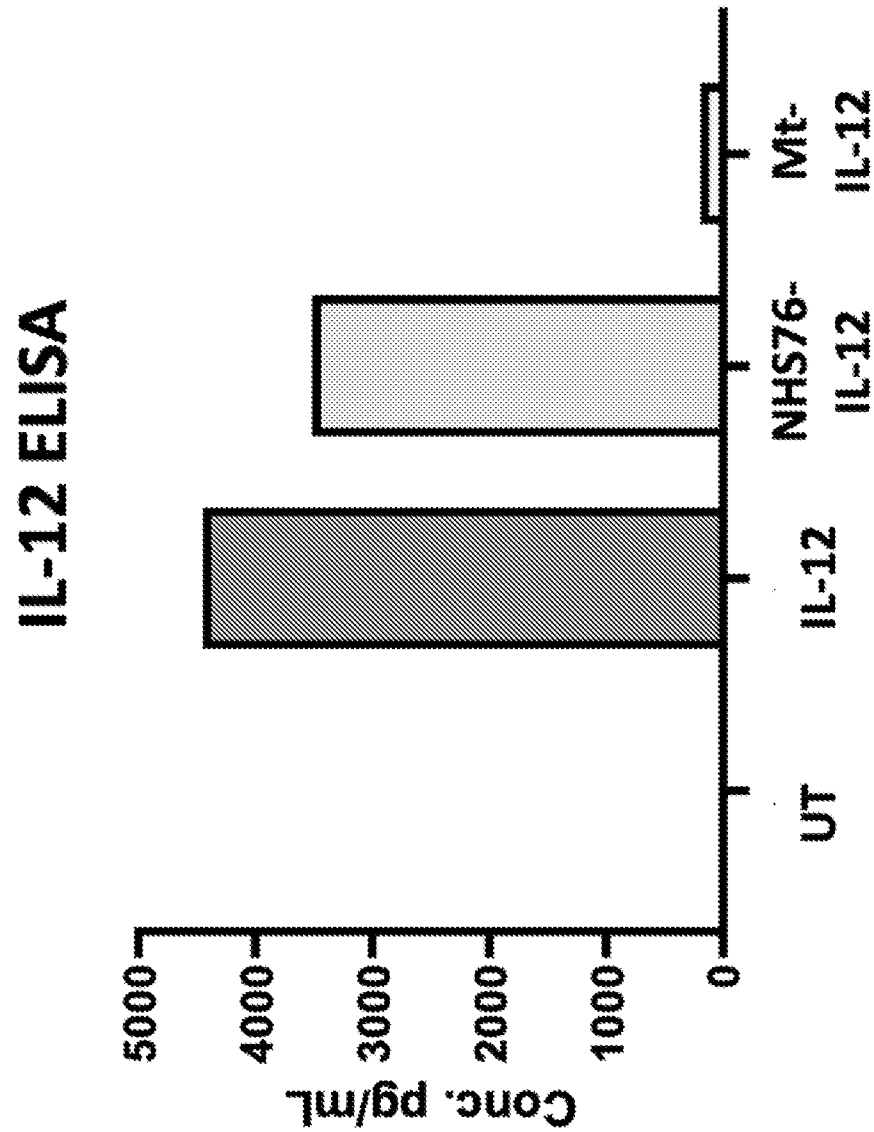


FIG. 16

FIG. 17

Mouse IL-12 construct amino acid sequence (SEQ ID NO: 1)

GSGATNFSLLKQAGDVEENPGPMCPQKLTISWFAIVLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLTCDTPEEDDITWTSDDQRHG
 VIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLLHKKENGIWSTEILKNFKNKTFLKCEAPNYSGRFTCSWLVRNMDLKFNKSS
 SSSPDSRAVTCGMASLSAEKVTLDQRDYEKYSVSCQEDVTCPTAEETLPIELALEARQNKYENYSTSFIRDIKPDPPKNLQMKPLK
 NSQVEVSWEYPDSWSTPHSYFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVQAQDRYVNSSCSKWACVPCRVR
 SGGGSGGGSGGGSRVIPVSGPARCLSQSRNLLKTDDMVKTAREKLEKHYSCATAEDIDHEDITRDQSTLTKTCLPLELHKNESECLAT
 RETSSTTRGSCLPQKTSLMMTLCLGSIYEDLKMYQTEFQAINAALQNHNHQQLILDKGMLVAIDELMQSLNHNGETLRQKPPVGEADP
 YRVKMKLCILLHAFSTRVVTINRVMGYLSSA

Mouse IL-12 construct nucleic acid sequence (SEQ ID NO: 2)

GGCAGCGGCGCCACCAACTTCAGCCTGCTTAACACAGGCGGACGCTGGAAGAGAAACCCCGGACCTATGTGCCCTCAGAAGCTGACCAT
 CAGTTGGTTCGCCATCGTGCTGCTGGTGTCCTCCACTGATGGCTATGTGGAGCTTGAGAAGGACGTGTACGTGGTGGAAAGTGACTGGA
 CCCCTGATGCTCCTGGCGAGACAGTGAACCTGACCTGGACACACCTGAAGAGGACGACATCACCTGGACCAGCGATCAGAGACACGGC
 GTGATCGGCTCTGGCAAGACCTGACAATTACCGTGAAGAGTTCCTGGACGCCGCCAGTACACCTGTACAAAGCGGAGAGACACT
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 GCGCAAGAAAGAAAAGATGAAGGAAACCGAGGAAGGCTGCAACCAAGAAAGGCGCTTTCCTGGTGGAAAAGACCAAGCCGAGGTGCAGT
 GCAAAGGCGGCAATGTCTGTGTGAGGCCCCAGGACCGGTACTACAACAGCAGCTGTAGCAAGTGGGCTGGTGGCCATGCAGAGTTAGA
 AGCGGAGGCGGAGGATCTGGTGGCGGAGGAAGTGGCGGAGGCGGTAGTAGAGTGATCCCTGTGTCTGGCCCTGCCAGATGTCTGAGCCA
 GAGCAGAAACCTGCTGAAAACCAACCGACGACATGGTCAAGACCGCCAGAGAGAAAGCTGAAGCACTACTCCTGCAAGCCGAGGACATCG
 ACCATGAGGACATCACAAAGGACCAAGCAAGCACCCCTGAAAACCTGCCCTGCTGGAACCTGCATAAGAACGAGAGAGTGCCTGGCCACC
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 GGATCTGAAGATGTACCAAGACCGAGTTCAGGCCATCAACGCCGCTCTCCAGAACCAACCAAGCAGAGATCATCTCTGGACAAGGGCA
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 TACAGAGTGAAGATGAAGCTGTGCATCCTGCTGCACGCCCTTACGACCCAGAGTGGTTCACCATCAACAGAGTGATGGGCTACCTGAGCAG
 CGCC

Mouse membrane-tethered IL-12 construct nucleic acid sequence (SEQ ID NO: 3)

GGCAGGGCGCCACCAACTTCAGCCTGCTTAAACAGGCGAGCGACGTGGAAGAGAACCCCGGACCTATGTGCCCTCAGAAAGCTGACCAT
 CAGTTGTTTCGCCATCGTGTGCTGCTGCCACTGATGGCTATGTGGAGCTTGAGAAAGACGTGTACGTGTGGAAAGTGGACTGGA
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 CGCGTGCCTAGAGATTGTGGCTGCAAGCCCTGCATCTGCACCGTGCCTGAAGTGTCCAGGTGTTTATCTTCCACCTAAGCCTAAGG
 ACGTGTGACAAATCACACTGACCCCTAAAGTGACCTGCGTGGTGGTGACATCTCCAAGGACGATCCCGAAGTGCAGTTCTCTTGGTTT
 GTGGACGACGTGGAAGTGCACACCGCTCAGACACAGCCAGAGAGGAACAGTTCAACAGCACCTTCAGAAAGCGTGTCCGAGCTGCCCCAT
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 ATGATCACCGATTCTTCCAGAGGACATTACCGTGGAATGGCAGTGGAAACGGCCAGCCTGCTGAGAAATTACAAGAAACACCCAGCCTAT
 CATGAACACCAACGGCTCCTACTTCGTGTACAGCAAGCTGAACGTGCAGAAAGTCTAACTGGAGGCGCGCAACACCTTCACATGCTCTG
 TGCTGCACGAGGACTGCACAATCACACACCGAGAAGTCCCTGTCTCACAGCCCTGGCAAGGTGTTCTCTGGCTTGTGTCTCGGCGGC
 AGCTTTGGCTTCCTGGGATTCTTCTGGGCTGTGTATCCTGTGC

Mouse membrane-tethered IL-12 construct amino acid sequence (SEQ ID NO: 4)

GSGATNFSLLKQAGDVEENPGMCPQKLTI SWFAI VLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLTCDTPEEDDITWTS DQRHG
 VIGSGKTLTI TVKEFLDAGQY TCHKGETLSHSHLL LHKKENG I WSTEI LKNFKNKTFLKCEAPNYSGRFTCSWL VQRNMDLKFNIKSS
 SSSPDSRAVTCGMASLSAEKVTL DQRDYEKYSVSCQEDVTCPTAEETLPIELALEARQQNKYENYSTSF FIRDI IKPDP PKNLQMKPLK

NSQEVSWSTPHSYFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVQAQDRYNNSSCSKWACVPCRVR
 SGGGGGGGGGGSRVIVPVGPARCLSQSRNLLKTDDVMKTAREKLKHYSCTAEDIDHEDITRDQSTLKTCLPLELHKNESCLAT
 RETSSTRGSCLPQKTSLSMTLCLGSIYEDLMYQTEFOAINAALQNHNHQOIILDKGMLVAIDELMQSLNHNGETLRQKPPVGEADP
 YRVKMKLCILLHAFSTRVVTINRVMGYLSSAVPRDCGCKPCICTVPEVSSVFIFFPKPKDVLITLTPKVTCVVVDISKDDPEVQFSWF
 VDDVEVHTAQTPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAFAPIEKTISKTKRPAKQVYTIPTPPKEQMAKDVKVSLTC
 MITDFFPEDI TVEWQWNGQPAENYKNTQPIMNTNGSYFVYSKLVNQSNWEAGNTFTCSVLHEGLHNHHTEKLSLHSPGKVFLACVLGG
 SFGFLGLGLCILC

Mouse long membrane-tethered IL-12 construct nucleic acid sequence (SEQ ID NO: 5)

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 CTCGCCGACGCCCCCGAGAAACAGTAAACTTGACATGTGACACCCCCGAGGAAGATGACATCACATGGACAAAGCACCAAGGCACGGC
 GTGATCGGTTTCAGGCAAGACCTCACTATTACAGTAAAGAGTTCTTGACGCGCGCCAGTATACATGCCATAAAGCGCGGAAACCTT
 GTCTCACTCCCATCTGCTTCCACAAAGAAAGACGGTATCTGGTCTACCGAAATCCTGAAAAATTTCAAAAAATAAACTTTCTCTGA
 AATGCGAAGCCCCCAATTACTCTGGGAGGTTTACCTGCTCATGGCTCGTTCAAAGAAATATGGATCTCAAATTCAAATTAAGTCTAGC
 TCCTCTAGTCCAGACTCTCGGGCAGTGACCTGTGGTATGGCTAGCCTTAGTGCAGAGAAGGTGACCCCTTGACCAACGAGATTATGAGAA
 GTACTCAGTGTCTTGTCAAGAAGACGTTACTTGTCCACAGCAGAAGAGACACTTCCCATCGAGTTGGCCCTTGAGGCCCGGCAGCAGA
 ACAAATATGAAAAATTATCAACTAGCTTTTATCCGCGACATCATAAACCCAGACCCCCCAAAAATTTGCAAAATGAAACCCACTGAAG
 AATAGCCAGGTGGAGTAAGCTGGAGTATCCAGATTCTTGGAGTACTCCACACAGTTACTTTAGCCTCAAAATTTTGTGAAGATACA
 AAGAAAGAAAGAAAAATGAAGGAAACTGAGGAGGTTGCAACCAAAAAGCGCAATTTCTTGTGGAGAAGACTAGTACAGAAGTCCAAT
 GTAAGGCGGGAATGTATGTGTCAAGCCCAGGATAGGTATTACAATTCAGTTCAGCAAAATGGCCTGCGTGCCATGTCGAGTTAGA
 TCCGGTGGGGCGGAAGCGGAGCGGTGGAAGCGGTGGAGGAGGTCAAGAGTAATACCTGTATCAGGGCCCCGACGATGCCTTTCTCA
 AAGCCGCAACTTGCTTAAGACAAACCGACGACATGGTTAAACAGCCCCGAGAAACTCAAGCACTATAGCTGTACCGCTGAGGACATCG
 ACCATGAGGATATCACAAAGGATCAACATCTACATTGAAAAACATGTCTGCCACTTGAGCTGCACAAAAATGAGAGTTGCTTGGCAACA
 CGGAAACCCAGCTCAACCCACAGAGGTCCTGTCTGCCCCCTCAAAAAACATCCTTGATGATGACACTCTGTCTTGGTTCATATACGA
 AGACTTGAAAAATGTACCAACACAGAGTTTCAAGCCATAAACGCACTCTCAAAAAACCAACCAACCAAAATAATCCTGGATAAGGGTA
 TGTGTTGTTGCAATAGATGAATTGATGCAATCTCTCAATCATAAATGGGAAACCTCAGGCAAAACCTCCTGTGGCGAGGCTGACCCA
 TACCGGTGAAGATGAAGTTGTGCATCCTGCTTCATGCATTTAGTACTCGGGTGGTAACTATCAACAGAGTAATGGGATACCTGAGTAG
 TGCCGTCCCCGGGATTGTGGCTGCAACCCCTGCATTTGTACAGTTCCAGAGGTTTCTTCCGTTTTCATTTTCCCCCTAAGCCTAAGG
 ACGTACTCACAAATTACCTCACACCTAAAGTAACATCGCTCGTGGTAGATATCAGTAAGGATGACCCAGAAGTGCAGTTTAGTTGGTTC
 GTTGACGACGTGGAGGTACATACCGCTCAGACCCAGCCGGAAGAACAGTTTAACTCCACTTCCGAAAGTTTTCAGAGCTTCCCAT
 CATGCATCAGGACTGGCTCAACGGCAAAGAGTTCAAATGTAGGTTCAACTCTGCAGCATTCCCAGCACCTATAGAGAAGACAATTTCTA
 AGACTAAAGTTCTTGAGGTGAGTGTGTTTCATTTTCCCTCCTAAACCTAAGGATGTCTTGACAAATCACTCTTACACCCAAAGTCACT

TGCGTTGTAGTTGACATTTCTAAAGATGACCCCTGAAGTTCAATTCTCATGGTTTGTGGATGACGTGGAGGTGCACACCGCACAAACTCA
ACCCCGCGAGGAGCAATTTAAATTTACATTTTCGGAGCGTTTCGGAGCTCCCAATCATGCACCAAGATTGGTTGAACGGCAAAAGAGTTCA
AGTGTGGGTCAATTCAGCTGCTTTTCAGCTCCTATTGAGAAAAACAATCTCTAAAAACAAAGGACGGCCCCAAAGCCCCCAGGTTTAT
ACAAATCCCACTCCTAAAGAGCAAAATGGCTAAGACAAGTCAAGTCTTACCTGTATGATCACCGATTTTTCCTCCGAGGACATAAAGTGT
TGAGTGGCAATGGAAACGGACAGCCTGCCGAAAACTACAAAAACACACAACCTATCATGAATACCAATGGCAGTTATTTTGTCTTACTCTA
AACTCAACGTGCAGAAGAGTAACCTGGGAGGCTGGGAATACTTTCACCTTGTAGTGTCTTCATGAAGGACTGCACAATCACCACACCGAG
AAGAGTCTTAGTCACTCCCCGGCAAAAGTTTCTCGCCTGCGTCTCTGGGCGGCTCATTTGGTTTCTCGGGTTTCTGGGCCCTGTGCAT
TCTTTGC

Mouse long membrane-tethered IL-12 construct amino acid sequence (SEQ ID NO: 6)

GSGATNFSLLKQAGDVEENPGMCPQKLTISWFAIVLLVSPMLAMWELEKDVYVVEVDWTPDAPGETVNLTCDTPEEDDITWTSQDRHG
 VIGSGKTLTITVKEFLDAGQYCHKGGETLSHSHLLHHKENGIWSTEILKNFNKNTFLKCEAPNYSGRFTCSWLVRQNMMDLKFNKISS
 SSSPDSRAVTCGMASLSAEKVTLDQRDYKEYSVSCQEDVTCPTAEETLPIELALEARQQNKYENYSTSFFIRDIKPDPPKNLQMKPLK
 NSQEVSWEYPDSWSTPHSYFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKSTEVQCKGGNVCVQAQDRYYNSSCSKWACVPCRV
 SGGGGGGGGGGGSRVIPVSGPARCLSQRNLLKTTDDMVKTAREKLKHYSCTAEDIDHEDI TRDQSTLTKCLPLELHKNESCLAT
 RETSSTTRGSCLPQKTSMMTCLGSIYEDLKMYQTEFQAINAALQNHNHQQIILDKGMLVAIDELMQSLNHNGETLRQKPPVGEADP
 YRVKMKLCILLHAFSTRVVTINRVMGYLSSAVPRDCGCKPCICTVPEVSSVFI FPPKPKDVLITLTLPKVTCVVVDISKDDPEVQFSWF
 VDDVEVHTAQTPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKVPEVSSVFI FPPKPKDVLITLTLPKVT
 CVVVDISKDDPEVQFSWFVDDVEVHTAQTPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVY
 TIPPPEQMAKDQVSLTCMITDFFPEDITVEQWQNGQPAENYKNTQPIMNTNGSYFVYSKLVNQKSNWEAGNTFTCSVLHEGLHNHHTTE
 KLSHSPGKVFLACVLGGSGFLGLGLCILC

Human IL-12 construct nucleic acid sequence (SEQ ID NO: 7)

GGATCTGGCGCCACC AATTTCAGCCTGCTGAAACAGGCTGGCGACGTGGAAGAGAAACCCCGGACCTATGTGTACCAAGCAGCTGGTCAT
CAGCTGGTTCAGCCTGGTGTTCCTGGCCTCTCCTGGTGGCCATCTGGGAGCTGAAGAAAGACGTGTACGTGGTGAAC TGACTGGT
ATCCCGATGCTCTTGGCGAGATGGTGTGACCTGCGATACCCCTGAAGAGGACGGCATCACCTGGACACTGGATCAGTCTAGCGAG
GTGCTCGGACGCGGCAAGACCTGACCATCCAAGTGAAGAGTTTGGCGACGCCGCCAGTACACCTGTCACAAAGCGGAGAAAGTGCT
GAGCCACAGTCTGCTGCTCCACAAGAAAGAGGATGGCATTTGGAGCACCGACATCTTGAAGGACCAGAAAGAGCCCAAGAACAAAGA
CCTTCTGAGATGCGAGGCCAAGAACTACAGCGGCCGGTTCACATGTTGGTGGCTGACCATCAGCACCGACCTGACCTTCAGCGTG
AAGTCCAGCAGAGGCAGCAGTGATCCTCAGGGCGTTACATGTGGCGCCGTACACTGTCTGCCGAAAGAGTGC GGGGCGACAAACAAGA
ATACGAGTACAGCGTGGAATGCCAAGAGGACAGCGCCTGTCCAGCGCCGGAAGAGTCTCTGCCCTATCGAAAGTATGTTGGACGCCGTGC
ACAAGCTGAAGTACGAGAACTACACCTCCAGCTTTTTCATCCGGGACATCATCAAGCCCGATCCTCCAAAAGAAATCTCCAGCTGAAGCCT
CTGAAGAACAGCAGACAGGTGGAAGTGTCTCTGGAGTACCCCGACACCTGGTCTACACCCACAGCTACTTCAGCCTGACCTTTTGCGGT

GCAAGTGCAGGGCAAGTCCAAGCGCGAGAAAAAGGACCGGGTGTTCACCGACAAGACCAGCGCCACCGTGATCTGCCGGAAGAAATGCCA
GCATCAGCGTCAGAGCCAGGACCGGTACTACAGCAGCTCTTGGAGCGAATGGCCAGCGTGCCATGTTCTGGTGGCGGAGGAAGCGGA
GGCGGAGGTTTCAAGCGCGGAGGATCAAGAAATCTGCCAGTGCCACGCGTGATCTTGCAATGTTCTTGTCTGCACCCACAGCCAGAA
CCTGCTGAGAGCCGTGTCCAACATGCTCCAGAAGGCCAGACAGACCCCTTGAGTTCTACCCCTGCACACGCGAGGAAATCGACACAGAGG
ACATCACCAAGGATAAGACCAGCACCGTGGAAGCTGCGTGCCTCTGGAACAGCAAGAACGAGAGTGCCTGAACAGCCGGGAACCC
AGCTTCATCACCAACGGCTCTTGCTGGCCAGCAGAAAGACCTCCTTCATGATGCCCCCTGTGCTGAGCAGCATCTACGAGGACCTGAA
GATGTACAGGTCGAGTTCAAGACCATGAACGCCAAGCTGCTGATGGACCCCAAGCGGAGATTTTCTGGACCAAGAAATATGCTGGCCG
TGATCGACGAGCTGATGCAGGCCCTGAACCTTCAACAGCGAGACAGTGCCTCCAGAAAGTCTAGCCTGGAAGAACCCGACTTCTACAAAGACC
AAGATCAAGCTGTGCATCCTGTGCACGCCCTTCCGGATCAGAGCCGTGACCATCGACAGAGTGATGACTACCTGAACGCCAGC

Human IL-12 construct amino acid sequence (SEQ ID NO: 8)

GGGATNFSLLKQAGDVEENPGPMCHQQLVISWFSLVFLASPLVAIWELKDDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSE
VLGSGKTLTIQVKEFGDAGQYTCCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSV
KSSRGSSDPQGVTCGAATLSAERVVRGDNKEYEYSVECEQEDSACPAAEESLPIEVMDAVHKLKYENYTSFFIRDIKPDPPKNLQLKP
LKNSRQVEVSWEPDWTSTPHSYFSLTFCVQVQKSKREKKDRVFTDKTSATVI CRKNAS I SVRAQDRYSSSWSEWASVPCSGGGGSG
GGSGGGGSRNLPVATPDPMFPCLHHSQNLRAVSNMLQARQTLFYPCTSEIDHEDI TKDKTSTVEACLPLELTKNESCLNSRET
SFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLIMDPKRQIFLDQNM LAVI DELMQALNFNSETVPPQKSSLEEPDFYKT
KIKLCILLHAFRIRAVTIDRVMSYLNAS

Human membrane-tethered IL-12 construct nucleic acid sequence (SEQ ID NO: 9)

GGATCTGGCGCCACCAATTTCAGCCTGCTGAAACAGGCTGGCGACGTGGAAGAGAACCCCGGACCTATGTGTACCAGCAGCTGGTCAT
CAGCTGGTTCAGCCTGGTGTTCCTGCGCTCTCCTCTGGTGGCCATCTGGAGCTGAAGAAAAGACGTGTACGTGGTGGAACCTGGACTGGT
ATCCCGATGCTCCTGGCGAGATGGTGGTGAACCTGCGATACCCCTGAAGAGGACGGCATCACCTGGACACTGGATCAGCTAGCGAG
GTGCTCGGACGGCAAGACCTGACCATCCAAGTAAAGAGTTTGGCGACCGCGCCAGTACACCTGTCAAAAGCGGAGAAAGTGCT
GAGCCACAGTCTGCTGCTCCACAAGAAAGAGGATGGCATTTGGAGACCCGACATCCTGAAGGACCAAGAAAGAGCCCAAGAAAGA
CCTTCTGAGATGCGAGGCCAAGAACTACAGCGGCCGGTTTACATGTTGGTGGCTGACCACCATCAGCACCGACCTGACCTTCAGCGTG
AAGTCCAGCAGAGGCAGCAGTGATCCTCAGGGCGTTACATGTGGCGCGCTACACTGTCTGCCGAAAAGAGTGGGGCGCAACAAGA
ATACGAGTACAGCGTGAATGCCAAGAGGACAGCGCTGTCCAGCGCGCGAAGAGTCTCTGCCATCGAAGTGTGGTGGACCGCGTGC
ACAAGCTGAAGTACGAGAACTACCTCCAGCTTTTCATCCGGGACATCATCAAGCCCGATCCTCCAAAGAAATCTCCAGCTGAAGCCT
CTGAAGAACAGCAGACAGTGGAAAGTGTCTGGGAGTACCCCGACACCTGGTCTACACCCACAGCTACTTCAGCCTGACCTTTTGGCT
GCAAGTGCAGGGCAAGTCCAAGCGCGAGAAAAGGACCGGTGTTCAACGACAAGACCAGCGCCACCGTGATCTGCCGGAAGAAATGCCA
GCATCAGCGTCAGAGCCAGGACCGGTACTACAGCAGCTCTTGGAGCGAATGGCCAGCGTGCCATGTTCTGGTGGCGGAGGAAGCGGA
GGCGGAGGTTCAAGCGCGGAGGATCAAGAAATCTGCCAGTGGCCACCGCTGATCCTGGCATGTTTCTTGTCTGCACCCACAGCCAGAA
CCTGCTGAGAGCCGTGTCCAACATGCTCCAAGAGGCCAGACAGACCTTGAGTTCTACCCCTGCACCGAGGAGGAAATCGACCCAGG

ACATCACCAAGGATAAGACCAGCACCGTGGAAGCCTGCCTGCCTCTGGAACCTGACCAAGAACGAGAGCTGCCTGAACAGCCGGGAAACC
 AGCTTCATCACCAACGGCTCTTGCTGGCCAGCAGAAAGACCTCCTTCATGATGGCCCTGTGCCTGAGCAGCATCTACGAGGACCTGAA
 GATGTACCAGGTCGAGTTCAGACCATGAACGCCAAGCTGCTGATGGAACCCCAAGGGCAGATTTCTTGACCAAGAATATGCTGGCCG
 TGATCGACGAGCTGATGAGGCCCTGAACCTCAACAGCGAGACAGTGCCCCAGAAAGTCTAGCCTGGAAGAACCCGACTTCTACAAAGACC
 AAGATCAAGCTGTGCATCCTGTGCACGCCCTTCCGGATCAGAGCCGTGACCATCGACAGAGTGATGAGCTACCTGAACGCCAGCGAGTC
 TAAGTACGGCCCTCCTTGCTAGTGGCCCGCTCCTGAATTTGAAGGGGCCCTTCCGTGTTCCGTGTTCCACCTAAGCCTAAGGACA
 CCCTGATGATCAGCAGAACACCCGAAGTGACCTGCTGGTGGACGTTTCAAGAGGACCCCGAGGTGCAAGTTAATTGGTACGTG
 GACGGCTGGAAGTGACAAACGCCAAGACAAAGCCAGAGAGGAACAGTTCCAGTCCACCTACAGAGTGGTGTCCGTGCTGACAGTGCT
 GCACCAAGGATGGCTGAACGGCAAGAGGTACAAGTGAAGGTTTCCAACAAGGCCCTGCCAAGCAGCATCGAGAAAACCATCTCCAAGG
 CCAAGGGCCAGCCTAGGGAACCCAGGTTTACACATGCTCCTCAAGCCAAAGAGATGACGAAGAATCAGGTGTCCCTGACCTGCCTG
 GTCAAGGGCTTTACCCAGCGACATTTGCCGTGGAATGGGAGAGCAATGGCCAGCCTGAGAACAACTACAAGACAACCCCTCCTGTGCT
 GGACAGCAGCGCTCATTTCTCTGTACTCCAGACTGACCGTGGACAAAGCAGATGGCAAGAGGGCAACGTGTTCAGCTGCTCCGTGA
 TGCACGAGGCCCTGCACAAACCATACACCCAGAAAAGCCTGAGCCTGTCTCTGGGCAAAATGGCCCTGATTGTGCTTGGCGAGTGGCC
 GGACTGCTGCTTTATCGGCCCTGGGCATCTTCTTC

Human membrane-tethered IL-12 construct amino acid sequence (SEQ ID NO: 10)

GS GATNFSLLKQAGDVEENPGPMCHQQLVLSWFSLVFLASPLVAIWELKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSE
 VLGS GKTLTIQVKEFGDAGQYTC HKGGEVLSHSLLLHKKEDGIWSTDILKDQKEPKNTFLRCEAKNYSGRFTCWMLTTISTDLTFSV
 KSSRGS SDPQGVTCGAATLSAERVGRDNKEYEYSVECEQDSACPAEEESLPIEVMDAVHKLKYENYTSFFIRDIKPDPPKNLQLKP
 LKNSRQVEVSWEPDTWSTPHSYFSLTFCVQVQKSKREKKDRVFTDKTSATVCRKNASISVRAQDRYSSSWSEWASVPCSGGGGSG
 GGGSGGGSRNLPVATPDGMFPCLHHSQNLRAVSNMLQARQTLFYPCTSEEDHEDI TKDKTSTVEACLPLELTKNESCLNSRET
 SFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVI DELMQALNFNSETVPQKSSLEEPDFYKT
 KIKLCILLHAFRIRAVTIDRVMSYLNASESKYGPSPCPAPEFEGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSQEDPEVQFNWYV
 DGVEVHNAKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTI SKAKGQPREPQVYTLPPSQEEMTKNQVSLTCL
 VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH EALHNHYTQKSLSLSLGKMALIVLGGVA
 GLLLFIGLGIFF

Human long membrane-tethered IL12 construct nucleic acid sequence (SEQ ID NO: 11)

GGATCTGGCGCCACCAATTT CAGCCTGCTGAAACAGGCTGGCGACGTGGAAGAGAACCCCGGACCTATGTGTACCAGCAGCTGGTCAT
 CAGCTGTT CAGCCTGGTGTTCCTGGCCTCTCCTCTGGTGGCCATCTGGAGCTGAAGAAAAGACGTGTACGTGTGGTGAACCTGGACTGGT
 ATCCCGATGCTCCTGGCGAGATGGTGTGCTGACCTGCGATACCCCTGAAGAGGACGGCATCACCTGGACACTGGATCAGTCTAGCGAG
 GTGCTCGG CAGCGGCAAGACCCCTGACCATCCAAGTGAAGAGTTTGGCGACGCCCGCCAGTACACCTGTCAAAAGCGGAGAAAGTGT
 GAGCCACAGTCTGCTGCTCCACAAGAAAGAGGATGGCATTTGGAGCACCGACATCTTGAAGGACCAGAAAAGAGCCCCAAGAACAGA
 CCTTCTGAGATGCGAGGCCAAGAACTACAGCGGCCGGTTT CACATGTTGGTGGCTGACCAACCATCAGCACCGACCTGACCTT CAGCGTG

AAGTCCAGCAGGCAGTGATCCTCAGGGCGTTACATGTGGCGCCGCTACACTGTCTGCCGAAAGAGTGGGGCGACAAACAAGA
ATACGAGTACAGCGTGGAAATGCCAAGAGGACAGCGCCTGTCCAGCCGCCGAAGAGTCTCTGCCCTATCGAAGTGATGGTGGACGCCGTGC
ACAAGCTGAAGTACGAGAACTACACCTCCAGCTTTTTCATCCGGGACATCATCAAGCCCGATCCTCCAAGAAATCTCCAGCTGAAGCCT
CTGAAGAACAGCAGACAGGTGGAAGTGTCTGGGAGTACCCCGACACCTGGTCTACACCCACAGCTACTTCAGCCTGACCTTTTGCCT
GCAAGTGCAGGGCAAGTCCAAGCGCGAGAAAAGGACCGGGTGTTCACCGACAAGACCGCCACCGTGATCTGCCGGAAGAAATGCCA
GCATCAGCGTCAGAGCCAGGACCGGTACTACAGCAGCTCTTGGAGCGAATGGGCCAGCTGCCATGTTCTGGTGGCGGAGGAAGCGGA
GGCGGAGTTCAGGCGGGAGGATCAAGAAATCTGCCAGTGGCCACGCTGATCTCTGGCATGTTCTTCTGTCTGCACCCACAGCCAGAA
CCTGCTGAGAGCCGTGTCCAACATGCTCCAAGGCGCAGACAGACCCCTTGAGTTCTACCCCTGCACCCAGCGAGGAAATCGACCACGAGG
ACATCACCAAGGATAAGACCAGCACCGTGAAGCCTGCCTGCCTCTGGAACGTGACCAAGAACGAGAGTGCCTGAACAGCCGGGAACCC
AGCTTCATCACCAACGGCTCTTGCTGGCCAGCAGAAAGACCTCCTTCATGATGCCCCGTGCCTGAGCAGCATCTACGAGGACCTGAA
GATGTACCAGGTCGAGTTCAAGACCATGAACGCCAAGCTGCTGATGGACCCCAAGCGGCAGATTTTCTGGACCAGAAATATGCTGGCCG
TGATCGACGAGCTGATGCAGGCCCTGAACCTCAACAGCGAGACAGTGCCCCAGAAAGTCTAGCCTGGAAGAACCCGACTTCTACAAAGACC
AAGATCAAGCTGTGCATCCTGTGCACGCCCTTCCGGATCAGAGCCCGTGACCATCGACAGAGTGATGAGCTACCTGAACGCCAGCGAGTC
TAAGTACGGCCCTCCTTGTCTAGTGCCTCCCGCTCCTGAATTTGAAGCGGCCCTTCCGTGTTCTGTTTCCACCTAAGCCTAAGGACA
CCCTGATGATCAGCAGAACACCCGAAGTGACCTGCTGGTGGACGTTTCAAGAGGACCCCGAGGTGCAGTTTAAATTTGTTACGTG
GACGGCTGGAAATGCACAAACGCCAAGACAAAGCCAGAGAGGAACAGTTCAGTCCACCTACAGAGTGGTGTCCGTGTGACAGTGCT
GCACCAGGATTTGGCTGAACGGCAAGAGTACAAGTGAAGGTTTCCAAACAAGGCCCTGCCAAGCAGCATCGAGAAAACCATCTCCAAGG
CCAAGGCTCCGAGTTCGAAGTGGCCCAAGTGTGTTCTGTCCCTCAAAAGCCAAAGGATACGCTCATGATCTCTCGACGCCCTGAA
GTGACATGTGCTGCTGATGTGTCCCAAGAAGATCCTGAAGTCCAGTTTAAATTTGATGTCGATGGCTCGAGGTCCACAATGCTAA
GACCAAGCCACGGGAAGAACAGTTTCAGAGCACCTACCGGGTGTGTCAGCTGCTCACTGTCTCATCAAGACTGGCTGAATGGGAAAG
AATACAAATGTAAGTGTCTAACAAAGGGCTCCCCAGTCCATCGAAAAGACAAATCAGCAAGGCCAAAGCCAGCCTCGCGAGCCTCAG
GTTTACACACTGCCTCCAAGCCAAAGAGAGATGACGAAGAATCAGGTGTCCCTGACCTGCTGGTCAAGGGCTTTTACCCAGCGACAT
TGCCGTGGAATGGGAGAGCAATGGCCAGCCTGAGAACTACAAGACAACCCCTCCTGTGCTGGACAGCGAGCTCATTTCTTCTGT
ACTCCAGACTGACCGTGGACAAGAGCAGATGGCAAGAGGGCAACGTGTTCAAGTGTCCGTGATGCACGAGGCCCTGCACAACCACTAC
ACCCAGAAAAGCCTGAGCCTGTCTCTGGGCAAAATGGCCCTGATTGTGCTTGGCGGAGTGGCCGACTGTGCTGTTTATCGGCCCTGGG
CATCTTCTTC

Human long membrane-tethered IL-12 construct amino acid sequence (SEQ ID NO: 12)

GS GATNFSLLKQAGDVEENPGPMCHQQQLVLSWFSLVFLASPLVAIWELKDDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSE
VLGSGKTLTIQVKEFGDAGQYTCCHKGGEVLSHSLLLHLKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWLTLTISTDLTFSV
KSSRGSDDPQGVTCGAATLSAERVVRGDNKEYEYSVEQCEDSACPAAFEESLPIEVMDVAVHKLKYNYSFFIRDI IKPDPPKLNQLKP
LKNSRQVEVSWEPDWTSTPHSYFSLTFCVQVQVQKSKREKKDRVFTDKTSATVICKRNASISVRAQDRYYSSSWSEWASVPCSGGGGSG
GGSGGGGSRNLPVATPDGMFPCLHHSQNLRLRAVSNMLQKARQTLFFYPCTSEEDHEDITKDKTSTVEACLPLEITKNESCLNSRET
SFITNGSCLASRKTSMFMALCLSSIIYEDLKMYQVEFKTMNAKLMLMDPKRQIFLDQNMLAVIDEIMQALNFNSETVPQKSSLEEDPFYKT

KIKLILLHAFRIRAVTIDRVMSYLNASESKYGPSPCPAPEFEGGPSVFLFPPKPKDILMI SRTPEVTCVVVDVSQEDPEVQFNWYV
 DGEVHNAKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTI SKAKAPEFEGGPSVFLFPPKPKDILMISRTPE
 VTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTI SKAKGQPREPQ
 VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVEFSCVMHEALHNHY
 TQKSLSLGKMALIVLGGVAGLLFLIGLIGFF

Human NHS76-IL-12 construct nucleic acid sequence (SEQ ID NO: 13)

GGATCTGGCGCCACCAATTTCAGCCTGCTGAACAGGCTGGCGACGTGGAAGAGAACCCCGGACCTATGTGTACACAGCAGCTGGTCAT
 CAGCTGGTTCAGCCTGGTTCCTGGCCTCTCCACTGGTGGCTCAGGTCAGCTGCAAGAGTCTGGCCCTGGCTGGTCAAGCCTAGCG
 AAACACTGAGCCTGACCTGTGCCGTGTCCGGCTACTCTATCAGCAGCGGCTATTACTGGGCTGGATCAGACAGCCTCCTGGCAAAGGC
 CTGGAATGGATCGCAGCATCTACACAGCGGACGACCTACTACAACCCAGCCTGAAGTCCAGAGTGACCATCAGCGTGGACACCCAG
 CAAGAACCCAGTTCTCCCTGAAGCTGAGCAGCGTGACAGCCGCGATACAGCCGTGTACTATTGCGCCAGAGGCAAGTGGTCCAAAGTTCCG
 ACTATTGGGGCCAGGCACCCCTGGTCACAGTTTCTAGCGGAGCGGAGGTTCTGTGGCGGAGGAAGTGGCGCGGAGGATCATCTTCT
 GAGTGACCCAAAGATCCTGGCGTGTCTGTGGCTCTGGGCCAGACAGTGGGATTACCTGTCAAGGCGATAGCCTGAGAAAGCTACTACGC
 CTCTGTGATCAGCAGAAAGCCTGGACAGGCTCCCGTGTCTGTGATCTACGGCAAGAACACAGACCCAGCGCATCCCCGATAGATTCA
 GCGGCTCTAGCTCTGGCAACACAGCCAGCCTGACAAATCACTGGCGCTCAGGCCGAAGATGAGCCGACTACTACTGCAACAGCAGAGAC
 AGTCCGGCAATCACGTGGTGTGGCGGAGGCACCAAGCTGACAGTTCTTGGAGGTGGTGTAGTGGCGGTGGTGGTTCAGGCGGTGG
 CGGATCTATTGGGAGCTGAAGAAAGACGTGTACGTGGTGGAACTGGACTGGTATCCCGATGCTCTGGCGAGATGGTGGTGTGACAT
 GCGATACCCCTGAAGAGCAGGATCACCTGGACACTGGATCAGTCTAGCGAGGTGCTCGGACGGCAAGACACTGACCATCCAAAGTG
 AAAGAGTTCGGCAGCGCGGCAGTACCTGTCAAAAGCGGAGAAAGTGTGAGCCACAGTCTGCTGTCTCCACAAGAAAGAGGA
 TGGCATTTGGAGCACCGACATCCTGAAGGACCAGAAAGAGCCCAAGAACCAAGACCTTCTTGAGATCGAGGCCAAGAACTACAGCGGCC
 GGTTACATGTTGGTGGCTGACCACTCAGCACCGACCTGACCTTCTCCGTGAAGTCTCCAGAGGCAGCAGTGATCCTCAGGGCGTT
 ACATGTGGCGCCGTACACTGTCTGCCGAAAGAGTGGCGGGCGACAAACAAAGAAATACGAGTACAGCTGGAATGCCAAGAGGACTCCGC
 CTGTCTCGCGCCGAAGAGTCTGTGCTATCGAAGTGATGGTGGACGCCGTGCACAAAGTGAAGTACGAGAACTACACCTCCAGCTTTT
 TCATCCGGGACATCATCAAGCCCGATCCTCCAAAGAAATCTCCAGCTGAAGCCTCTGAAGAACAGCAGACAGGTGGAAGTGTCTCTGGAG
 TACCCCGACACCTGGTCTACACCCACAGCTACTTTAGCCTGACATTTGCGTGAAGTCAAGTCAAGGCAAGTCCAAAGCGGAGAAAAAGGA
 CCGGGTGTTCACCGACAAGACCGCCACCGTGATCTGTAGAAAGAACCCAGCATCAGCGTCAGAGCCAGGACCGGTACTACAGCA
 GCTCTGGAGCGGAATGGGCCAGCTGCCATGTAGTGGCGGAGGGCGCAGCGGGAGGGGATCTGGCGGTGGTGAAGTAGAAATCTG
 CCCGTGGCCACTCCTGATCCTGGCATGTTTCTTGGCTGCACACAGCCAGAACCTGTGTGAGAGCCGTGTCCAACATGCTCCAGAAAGGC
 CAGGCAGACCCCTTGAGTTCTACCCCTGCACACGAGGAGGAATCGACACGAGGACATCACCAAGGATAAGACACGACCCGTGGAAGCCT
 GCCTGCCACTGGAACAGACGAGAGTGCCTGAACAGCCGGGAAACCAAGCTTCATCACCAACGGCTCTTGGCTGGCCAGCAGA
 AAGACCTCCTTCATGATGGCCCTGTGTCTGTCTCCATCTACGAGGACCTGAAGATGTACCAGGTGAGTTCAGACCATGAACGCCAA
 GCTGCTGATGGACCCCAAGCGGACAGATTTTCTCTGGACCAGAACATGCTGGCCGTGATCGACGAACATGATCGAGGCCCTGAACCTCAACT

CCGAGACAGTGCCCCAGAAAGTCCAGCCTGGAAGAACCCGACTTCTACAAGACCAAGATCAAGCTGTGCATCCTGCTGCACGCCTTCCGG
ATCAGAGCCGTGACCATCGACAGAGTGATGAGCTACCTGAACGCCTCC

Human NHS76-IL-12 construct amino acid sequence (SEQ ID NO: 14)

GS GATNFSLLKQAGDVEENPGPMCHQQLVLSWFSLVFLASPLVAQVQLQESGPGLVKPSSETLSLTCAVSGYSISSGYYWGWI RQPPGKG
LEWIGSIYHSGSTYYNPSLKSRVTISVDTSKNQFSLKLSVTAADTAVYVCARGKWSKFDYWGQTLVTVSSGGGGGGGGGGSSSS
ELTQDPAVSVALGQTVRITCQGDLSRYYASWYQQPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTIITGAQAEDEADYYCNSRD
SSGNHVFFGGGTKLTVLGGGGGGGGGGGGSIWELKDVYVVELDWYDPAGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLTIQV
KEFGDAQYTCHKGGEVLSHLLHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWMLTTISTDLTFSVKSSRGSSDDPQGV
TCGAATLSAERVVRGDNKEYEYSVECEQDSACPAAEESLPIEVMDAVHKLKYENYTSFFIRDIKPDPPKNLQLKPLKNSRQVEVSWE
YPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCSGGGGGGGGGGGSRNL
PVATPDGMPFCLHHSQNLRAVSNMLQKARQTLFYPCTSEEDHEDITKDKTSTVEACLPLELTKNESCLNSRETSTFTNGSCLASR
KTSFMMALCLSSIYEDLKMYYQVEFKTMNAKLMDPKRQIFLDQNM LAVIDE LMQALNFNSETVPPQKSSLEEPDFYKTKIKLCILLHAFR
IRAVTIDRVMSYLNAS

Human IL-12 alpha subunit amino acid sequence (P35) (SEQ ID NO: 15)

RNLPVATPDGMPFCLHHSQNLRAVSNMLQKARQTLFYPCTSEEDHEDITKDKTSTVEACLPLELTKNESCLNSRETSTFTNGSCL
ASRKTSFMMALCLSSIYEDLKMYYQVEFKTMNAKLMDPKRQIFLDQNM LAVIDE LMQALNFNSETVPPQKSSLEEPDFYKTKIKLCILLH
AFRIRAVTIDRVMSYLNAS

Human IL-12 beta subunit amino acid sequence (P40) (SEQ ID NO: 16)

IWELKKDVYVVELDWYYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYCHKGGEVLSHLLHKKEDGI
WSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWMLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERVVRGDNKEYEYSVECEQDSACP
AAEESLPIEVMDAVHKLKYENYTSFFIRDIKPDPPKNLQLKPLKNSRQVEVSWEYPTWTSTPHSYFSLTFCVQVQGKSKREKKDRV
FTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCS

Linker peptide sequence (SEQ ID NO: 17)

GGGGGGGGGGGGGG

Linker peptide sequence (SEQ ID NO: 18)

GGGGGGGGGG

Linker peptide sequence (SEQ ID NO: 19)

GGGGSGGGSGGGSGGGGS

Linker peptide sequence (SEQ ID NO: 20)
GGGS

Human IL-12 signaling peptide amino acid sequence (SEQ ID NO: 21)
MCHQQLVISWFLVFLASPLVA

EBV LMP2 TCR L201 Va (SEQ ID NO: 22)
DQQVKQNSPSLSVQEGRISILNCDYTNMFDYFLWKYPAEGPTFLISSIKDKNEDGRFTVFLNKS AKHLSLHIVPSQPGDSAVYF
CAAPPPSGNQFYFGTGTSLTVIP

EBV LMP2 TCR L201 Vb (SEQ ID NO: 23)
DVKVTQSSRYLVKRTGEKVLECVQDMDHENMFWYRQDPGLRLIYFSYDVKMKEKGDIPEGYSVSREKKERFSLILESASTNQTSMY
LCASSTRLAGAQRDTQYFGPGTRLTVL

EBV LMP2 TCR L202 Va (SEQ ID NO: 24)
EDVEQSLFSLVREGDSSVINCTYTDSSSTLYWYKQEPGAGLQLLTYIFSNMDMKDQRLTVLLNKKDKHLSLRIADTQTGDSAIYFCA
ESIPSGYNKLIIFGAGTRLAVHP

EBV LMP2 TCR L202 Vb (SEQ ID NO: 25)
GVAQSPRYKIEKRQSVAFWCNPISGHATLYWYQQLLGQPKLLIQFNNGVVDDSQLPKDRFSAERLKGVDSTLKIQPAKLEDSAVYL
CASSLGLAGEDTQYFGPGTRLTVL

EBV LMP2 TCR L203 Va (SEQ ID NO: 26)
QQKNDDQQVKQNSPSLSVQEGRISILNCDYTNMFDYFLWKYPAEGPTFLISSIKDKNEDGRFTVFLNKS AKHLSLHIVPSQPGD
SAVYFCAAKLPQAAGNKLTFFGGTRVLVKP

EBV LMP2 TCR L203 Vb (SEQ ID NO: 27)
MVIQNPYQVTQFGKPVTLSCSQTLNHNVMYWYQQKSSQAPKLLFHYDYKDFNNEADTPDNFQSRRPNTSFCFLDIRSPGLGDAAMYLC
ATSSSIAGDTRNNEQFFGPGTRLTVL

HPV E6 TCR Va (SEQ ID NO: 28)

MLLEHLLIIL WMQLTWVSGQ QLNQSPQSMF IQEGEDVSMN CTSSSIFNTW LWYKQDPGEG PVLLIALYKA GELTSNGRLT
 AQFGITRKDS FLNISASIPS DVGIFYFCAGH PSSNSGYALN FGKGTSLT P

HPV E6 TCR Vb (SEQ ID NO: 29)
MGTSLLCWV LGFLGTDHTG AGVSQSPRYK VTKRGQDVAL RCDPISGHVS LYWYRQALGQ GPEFLTIFYNY EAQQDKSGLP
 NDRFSAERPE GSISTLTIOR TEQRDSAMYR CASSQTGAR TDTQYFGPGT RLTVL

HPV E7 TCR Va (SEQ ID NO: 30)
 MWGVFLLYVS MKMGTTGQN IDQPTMTAT EGAIVQINCT YQTSGFNGLF WYQQHAGEAP TFLSYNVLDG LEEKGRFSSF
 LSRKGYSYL LLKELQMKDS ASYLCASVDG NNRLAFGKGN QVVVIP

HPV E7 TCR Vb (SEQ ID NO: 31)
 MXPGLLCWAL LCLLGAGLVD AGVTQSPTHL IKTRGQQVTL RCSPKSGHDT VSWYQQALGQ GPQFIFQYEE EERQRGNFP
 DRFSGHQFPN YSSEINVNAL LLGDSALYLC ASSLGWRGGR YNEQFFGPGT RLTVL

HPV E6 TCR E202 Va (SEQ ID NO: 32)
 AQTVTQSQPEMSVQEAETVTLSTYDTSESDDYILFWYKQPPSRQMLVIRQEAAYKQQNATENRFSVNFQKAAKSFSLKISDSQLGDAAM
 YFCAYRDSRGSTLGRLYFGRGTQLTVWP

HPV E6 TCR E202 Vb (SEQ ID NO: 33)
 DVKVTQSSRYLVKRTGEKVFLECVQMDMDHENMFWYRQDPGLGLRLIYFSYDVKMKKEKGDIPGYSVSREKKERFSLILESASTNQTSMY
 LCASSLWGRKNTEAFFGQGRRLTVV

NY-ESO-1 TCR N201 Va (USC) (SEQ ID NO: 34)
 GQQLNQSPQSMFIQEGEDVSMNCTSSSIFNTWLWYKQDPGEGPVLLIALYKAGELTSNGRLTAQFGITRKDSFLNISASIPSDVGIYFC
 AGFLDSNYQLIWGAGTKLIIKPD

NY-ESO-1 TCR N201 Vb (USC) (SEQ ID NO: 35)
 EAQVTQNPRYLITVTGKKLTVTCSQNMNHEYMSWYRQDPGLGLRQIYYSMNVEVTDKGDVPEGYKVSKEKRNFPILIESPSPNQTSLY
 FCASASGYRTDTQYFGPGTRRLTVLED

CD19 CAR scFv (SEQ ID NO: 36)

mewtwvflfl lsvtagvhds i qmtqtssl saslgdrvti scrasqdisk
 ylnwyqqkpd gtvkllyht srlhsgvpsr fsgsgsgtdy sltisnleqe
 diatyfcqgg ntlpytfggg tkleitgsts gsgkpgsgeg stkgevklqe
 sgpglvapsq slsvtctvsg vsldygvsw irqpprkgle wlgviwgset
 tyynsalksr ltiikdnks qvflkmnslq tddtaiyyca khyyyggsya
 mdwygqgtsv tvsshhhhhh

BCMA CAR (SEQ ID NO: 37)

MALPVTALLL PLALLHAAR PDIVLTQSP SLAMSLGKRA TISCRASESV
 TILGSHLIHW YQKPGQPPT LLIQLASNVQ TGVPARFSGS GSRTDFTLTI
 DPVEEDDVAV YYCLQSR TIP RTFGGGTKLE IKGSTSGSK PGSGEGSTKG
 QIQLVQSGPE LKKPGETVKI SCKASGYTFT DYSINWVKRA PGKGLKWMGW
 INTETREPAY AYDFRGRFAF SLETSASTAY LQINNLYED TATYFCALDY
 SYAMDYWGQG TSVTVSSAAA TTPAPRPPT PAPTIASQPL SLRPEACRPA
 AGGAVHTRGL DFACDIYIWA PLATCGVLL LSLVITLYCK RGRKKLLYIF
 KQPFMRPVQT TQEDGCSGR FPEEEGGCE LRVKFSRSAD APAYQQGQNG
 LYNELNLGR EYDVLDKRR GRDPEMGKPK RRKNPQEGLY NELQDKMAE
 AYSEIGMKE RRRGKGHDGL YQGLSTATKD TYDALHMQAL PPR

BCMA CAR (SEQ ID NO: 38)

MALPVTALLL PLALLHAAR PVNRTRRMHQ VQLVESGGGL VQPGGSLRLS
 CEASGFTLDY YAIGWFRQAP GKREGVICI SRSDGSTYYA DSVKGRFTIS
 RDNAKKTVYL QMISLKPEDT AAYYCAAGAD CSGYLRDYEY RGQGTQVTVS
 STSTTPAPR PTPAPTAS QPLSLRPEAC RPAAGGAVHT RGLDFACDFW
 VLVVVGGLA CYSLLVTVAF IIFWVRSKRS RLLHSDYMN TPRRPGPTRK
 HYQPYAPPRD FAAYRSKRGR KLLYIFKQF FMRPVQTTQE EDGSCRFPE
 EEEGGCEL RV KFSRSADAPA YKQGNQLYN ELNLGRREY DVLDKRRGRD
 PEMGGKPRK NPQEGLYNEL QDKMAEAYS EIGMKGERRR GKGDGLYQG
 LSTATKDTYD ALHMQALPPR

BCMA CAR FN3 domain (SEQ ID NO: 39)

MLPAPKNLVV SRITEDSARL SWTAPDAAFD SFPIRYIETL IWGEAIWLDV
 PGSERSYDLT GLKPGTEYTV VIDGVKGGGR SQPLVATFTT

IL13Ra2 CAR (SEQ ID NO: 40)

GPVPPSTALRYLIEELVNITONQKAPLCNGSMVWSINLTAGMYCAALESLINVSGCSAIEKQTQRMLSGFCPHKVSAGQFSSLHVRDTKI
EVAQFVKDLLLLHLKKLFREGFRN

Human mutated CH2 (hmCH2) amino acid sequence (SEQ ID NO: 41)

APEFEGGPSVFLFPPKPKDTLMI SRTPEVT CVVVDVSQEDPEVFQFNWYVDGVEVHNAKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKGLPSSIEKTIISKAK

Human mutated CH2 (hmCH2) nucleic acid sequence (SEQ ID NO: 42)

GCTCCTGAATTTGAAGCGGCCCTTCCGTGTTCCGTGTTCCACCTAAGCCTAAGGACACCCCTGATGATCAGCAGAACACCCGAAGTGAC
CTGCGTGGTGGTGGACGTTTCACAAAGAGGACCCCGAGGTGCAGTTTAATTGTTACGTGGACGGCGTGGAAGTGCACAACGCCAAGACAA
AGCCCAAGAGAGGAACAGTTCAGTCCACCTACAGAGTGGTGTCCGTGCTGACAGTGTGACCCAGGATTGGCTGAACGGCAAAGAGTAC
AAGTGCAAGGTTTCCCAACAAGGGCCTGCCAAGCAGCATCGAGAAAACCATCTCCAAAGGCCAAG

Human CH3 (hCH3) amino acid sequence (SEQ ID NO: 43)

GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH
EALHNHYTQKSLSLIGK

Human CH3 (hCH3) nucleic acid sequence (SEQ ID NO: 44)

GGCCAGCCTAGGGAACCCAGGTTTACACACTGCCCTCCAAGCCCAAGAAAGAGATGACGAAGAATCAGGTGTCCCTGACCTGCCTGGTCAA
GGGCTTTACCCCAAGCGACATTGCCGTGGAATGGGAGAGCAATGGCCAGCCTGAGAACAACACTACAAGACAACCCCTCCTGTGTGGACA
GGACGGCTCATTTCTTCTGTACTCCAGACTGACCGTGGACAAAGAGCAGATGGCAAAGAGGGCAACGTGTTTCAGCTGCTCCGTGATGCAC
GAGGCCCTGCACAACCACTACACCCAGAAAAGCCTGAGCCTGTCTCTGGGCAAA

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2020/090885

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/705(2006.01)i; C07K 14/725(2006.01)i; C07K 14/54(2006.01)i; A61K 38/20(2006.01)i; A61K 39/395(2006.01)i

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)

C07K; A61K

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)

CNABS, SIPOABS, DWPI, CNTXT, WOTXT, USTXT, EPTXT, CNKI, WANGFANG Database, PubMed, ISI Web of Knowledge, Baidu Xueshu: TCRCURE, IL-12, interleukin-12, TCR, CAR, NHS-76, transmembrane domain, membrane bounded/tethered/anchored, CH2, CH3, TM, CD4, immunoglobulin, constant region

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KONERU, M. et al. "IL-12 secreting tumor-targeted chimeric antigen receptor T cells eradicate ovarian tumors in vivo" <i>Onc Immunology</i> , Vol. 4, No. 3, 16 March 2015 (2015-03-16), e994446, pages 1-11	1, 10, 14-16, 39-41, 49-81
Y	KONERU, M. et al. "IL-12 secreting tumor-targeted chimeric antigen receptor T cells eradicate ovarian tumors in vivo" <i>Onc Immunology</i> , Vol. 4, No. 3, 16 March 2015 (2015-03-16), e994446, pages 1-11	11-13, 34-36
X	ZHANG, L. et al. "Enhanced efficacy and limited systemic cytokine exposure with membrane-anchored interleukin-12 T-cell therapy in murine tumor models" <i>Journal for ImmunoTherapy of Cancer</i> , Vol. 8, 19 January 2020 (2020-01-19), e000210, pages 1-12	1-9, 14-28, 39-43, 46-81
Y	ZHANG, L. et al. "Enhanced efficacy and limited systemic cytokine exposure with membrane-anchored interleukin-12 T-cell therapy in murine tumor models" <i>Journal for ImmunoTherapy of Cancer</i> , Vol. 8, 19 January 2020 (2020-01-19), e000210, pages 1-12	11-13, 34-36



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

26 January 2021

Date of mailing of the international search report

20 February 2021

Name and mailing address of the ISA/CN

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2020/090885

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015124297 A1 (MERCK PATENT GMBH) 27 August 2015 (2015-08-27) description page 3 last paragraph	29-33, 37-38, 44-70
Y	WO 2015124297 A1 (MERCK PATENT GMBH) 27 August 2015 (2015-08-27) description page 3 last paragraph	11-13, 34-36
A	WO 2016172703 A2 (HAEMALOGIX PTY. LTD. ET AL.) 27 October 2016 (2016-10-27) the whole document	1-81
A	KONERU, M. et al. "A phase I clinical trial of adoptive T cell therapy using IL-12 secreting MUC-16ecto directed chimeric antigen receptors for recurrent ovarian cancer" <i>Journal of Translational Medicine</i> , Vol. 13, No. 102, 28 March 2015 (2015-03-28), pages 1-11	1-81
A	WO 2017062953 A1 (INTREXON CORP.) 13 April 2017 (2017-04-13) the whole document	1-81
A	ECKERT, F. et al. "Tumor-targeted IL-12 combined with local irradiation leads to systemic tumor control via abscopal effects in vivo" <i>Oncotarget</i> , Vol. 6, No. 6, 14 June 2017 (2017-06-14), e1323161, pages 1-12	1-81
A	PAN, W. Y. et al. "Cancer Immunotherapy Using a Membrane-bound Interleukin-12 With B7-1 Transmembrane and Cytoplasmic Domains" <i>Molecular Therapy</i> , Vol. 20, No. 5, 14 February 2012 (2012-02-14), pages 927-937	1-81
A	FALLON, J. et al. "The immunocytokine NHS-IL12 as a potential cancer therapeutic" <i>Oncotarget</i> , Vol. 5, No. 7, 24 March 2014 (2014-03-24), pages 1869-1884	1-81

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2020/090885

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

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

1. ☒ Claims Nos.: **67-81**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] The subject matter of claims 67-81 relates to methods of treating a subject having a cancer, and therefore does not warrant an international search according to the criteria set out in Rule 39.1(iv). However, the search has been carried out and based on the use for manufacturing a medicament.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2020/090885

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2015124297	A1	27 August 2015	PT	3107573	T	10 January 2019
				EP	3107573	A1	28 December 2016
				RU	2016136990	A	22 March 2018
				RU	2689160	C2	24 May 2019
				CA	2940018	A1	27 August 2015
				AU	2015221181	B2	20 August 2020
				RU	2016136990	A3	10 October 2018
				AU	2015221181	A1	29 September 2016
				KR	20160120335	A	17 October 2016
				CN	105992590	B	31 December 2019
				EP	3107573	B1	26 September 2018
				JP	6416926	B2	31 October 2018
				CN	105992590	A	05 October 2016
				MX	2016010674	A	08 November 2016
				CA	2940018	C	04 December 2018
				JP	2017507937	A	23 March 2017
				US	2017166620	A1	15 June 2017
				ES	2703826	T3	12 March 2019
WO	2016172703	A2	27 October 2016	CN	107922489	A	17 April 2018
				AU	2016253126	B2	30 January 2020
				US	2018228892	A1	16 August 2018
				EP	3286223	A2	28 February 2018
				JP	2018519356	A	19 July 2018
				EP	3286223	A4	05 December 2018
				BR	112017022838	A2	17 July 2018
				RU	2017138528	A3	03 September 2019
				RU	2017138528	A	07 May 2019
				AU	2016253126	A1	12 October 2017
				KR	20170139044	A	18 December 2017
				HK	1252854	A1	06 June 2019
				IL	255207	D0	31 December 2017
				WO	2016172703	A3	24 November 2016
				CA	2978964	A1	27 October 2016
				HK	1251586	A1	01 February 2019
WO	2017062953	A1	13 April 2017	AU	2016334401	A1	26 April 2018
				SG	11201802912P	A	30 May 2018
				CA	3001590	A1	13 April 2017
				EP	3347373	A1	18 July 2018
				US	2019062394	A1	28 February 2019