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(71) Applicant: **PACT PHARMA, INC.** [US/US]; 2 Corporate Drive, South San Francisco, CA 94080 (US).

(72) Inventors: **SENNINO, Barbara**; c/o PACT PHARMA, INC., 2 Corporate Drive, South San Francisco, CA 94080 (US). **JACOBY, Kyle**; c/o PACT PHARMA, INC., 2 Corporate Drive, South San Francisco, CA 94080 (US). **MANDL-CASHMAN, Stefanie**; c/o PACT PHARMA, INC., 2 Corporate Drive, South San Francisco, CA 94080 (US). **DUBREUIL, Michael, M.**; c/o PACT PHARMA, INC., 2 Corporate Drive, South San Francisco, CA 94080 (US). **GAGNON, John**; c/o PACT PHARMA, INC., 2 Corporate Drive, South San Francisco, CA 94080 (US). **FRANZUSOFF, Alex**; c/o PACT PHARMA, INC., 2 Corporate Drive, South San Francisco, CA 94080 (US).

(74) Agent: **LENDARIS, Steven, P.** et al.; Baker Botts LLP, 30 Rockefeller Plaza, New York, NY 10112-4498 (US).

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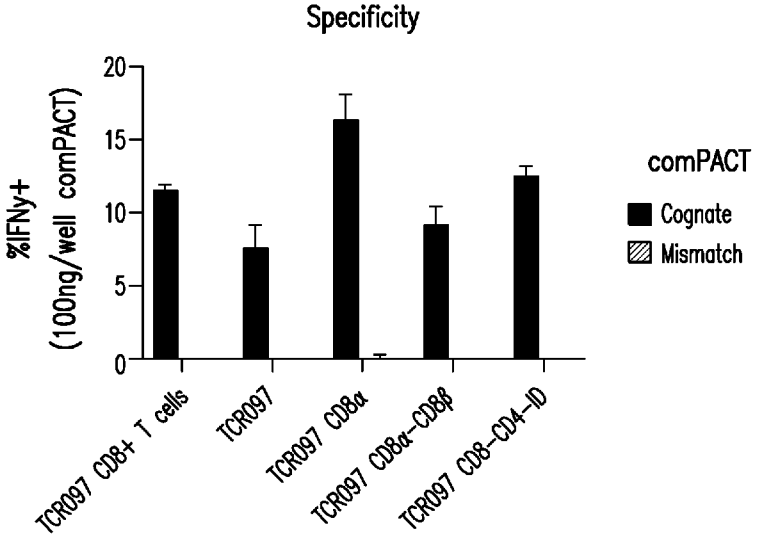


FIGURE 17B

(57) Abstract: Compositions comprising and methods for the treatment of cancer using a neoTCR based cell therapy with a CD8 expression construct. In certain embodiments, the presently disclosed subject matter provides a cell, comprising an exogenous T cell receptor (TCR), and an exogenous CD8. In certain embodiments, the exogenous CD8 comprises at least one monomer. In certain embodiments, the at least one monomer of the exogenous CD8 comprises an extracellular domain, a transmembrane domain, an intracellular domain, fragments thereof, or combinations thereof.

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COMPOSITIONS AND METHODS FOR THE TREATMENT OF CANCER USING A CD8 ENGINEERED T CELL THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/841,748, filed on May 1, 2019, and U.S. Provisional Application No. 62/841,753, filed on May 1, 2019, the content of which are incorporated in their entirety, and to which priority is claimed.

SEQUENCE LISTINGS

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on April 30, 2020, is named 087520_0145_SL.txt and is 120,294 bytes in size.

BACKGROUND OF THE INVENTION

Activation of T cells requires signaling through the T cell receptor (TCR) and its coreceptor molecules. CD4 and CD8 are membrane proteins that are expressed on T helper cells and cytotoxic T lymphocytes that serve as coreceptors that augment TCR signaling by stabilizing the interactions between the peptide-major histocompatibility (pMHC) ligands and the TCR (Li QJ, et al. (2004) CD4 enhances T cell sensitivity to antigen by coordinating Lck accumulation at the immunological synapse. Nat Immunol 5:791–799; Holler PD, Kranz DM (2003) Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. Immunity 18:255–264). Specifically, the CD4 and CD8 coreceptors are essential for the initiation of signaling because they facilitate the recruitment of a kinase to the TCR-pMHC complex. Furthermore, research has shown that while the CD4 and CD8 coreceptors both augment T cell sensitivity to its ligands, only CD8 plays a role in the stabilization of TCR-pMHC interactions.

Furthermore, while naturally occurring MHC-I TCRs are presumed to require concurrent CD8 co-receptor help to stabilize peptide-MHC binding, higher affinity TCRs drive CD8-independent target binding and T cell activation. CD4 T cells, when engineered with high affinity NeoTCRs, are thus able to recognize peptide-MHC-I targets and trigger

effector T cell functions. However, lower affinity TCRs are dependent on CD8 co-receptors to trigger T cell activation.

Accordingly, a NeoTCR cell therapy that is engineered to have CD8 co-receptor expression could stabilize the TCR-pMHC interactions and increase the efficacy of NeoTCR cell therapies that comprise low affinity TCRs that are dependent on such CD8 co-receptors.

SUMMARY OF THE INVENTION

In certain embodiments, the presently disclosed subject matter provides a cell, comprising an exogenous T cell receptor (TCR), and an exogenous CD8. In certain embodiments, the exogenous CD8 comprises at least one monomer. In certain embodiments, the at least one monomer of the exogenous CD8 comprises an extracellular domain, a transmembrane domain, an intracellular domain, fragments thereof, or combinations thereof. In certain embodiments, the extracellular domain comprises a CD8α extracellular domain or a CD8β extracellular domain. In certain embodiments, the transmembrane a CD8α transmembrane domain or a CD8β transmembrane domain. In certain embodiments, the intracellular domain comprises a CD8α intracellular domain or a CD8β intracellular domain. In certain embodiments, the intracellular domain comprises a CD4 intracellular domain.

In certain embodiments, the at least one monomer comprises a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain. In certain embodiments, the at least one monomer comprises a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain. In certain embodiments, the at least one monomer comprises a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain. In certain embodiments, the at least one monomer comprises a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain. In certain embodiments, the at least one monomer comprises a signal peptide. In certain embodiments, the signal peptide is a CD8 signal peptide.

In certain embodiments, the extracellular domain comprises the amino acid sequence set forth in SEQ ID NO: 140, or SEQ ID NO: 145. In certain embodiments, the transmembrane domain comprises the amino acid sequence set forth in SEQ ID NO: 141, or SEQ ID NO: 146. In certain embodiments, the intracellular domain comprises the amino acid sequence set forth in SEQ ID NO: 142, SEQ ID NO: 147, or SEQ ID NO: 148.

In certain embodiments, the signal peptide comprises the amino acid sequence set forth in SEQ ID NO: 139, or SEQ ID NO: 144.

In certain embodiments, the exogenous CD8 comprises a 2A sequence. In certain embodiments, the exogenous CD8 comprises a linker. In certain embodiments, the linker comprises the amino acid sequence set forth in SEQ ID NO: 137. In certain embodiments, the exogenous CD8 comprises a protease cleavage site. In certain embodiments, the protease cleavage site is a Furin cleavage site.

In certain embodiments, the exogenous TCR is a patient derived TCR. In certain embodiments, the exogenous TCR comprises a signal sequence, a first and second 2A sequence, and a TCR polypeptide sequence. In certain embodiments, the exogenous TCR recognizes a cancer antigen. In certain embodiments, the cancer antigen is a neoantigen. In certain embodiments, the cancer antigen is a patient specific antigen.

In certain embodiments, the cell is a primary cell. In certain embodiments, the cell is a patient-derived cell. In certain embodiments, the cell is a lymphocyte. In certain embodiments, the cell is a T cell. In certain embodiments, the cell if a young T cell. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+. In certain embodiments, the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

In certain embodiments, the cell further comprises a gene modification to enhance cell persistence and/or enhances memory cell differentiation. In certain embodiments, killing activity of the cell is increased between about 10% to about 500% as compared to killing activity of a cell that does not have the exogenous CD8. In certain embodiments, proliferation of the cell upon binding of the TCR to the antigen is increased between about 10% to about 500% as compared to proliferation of a cell that does not have the exogenous CD8. In certain embodiments, secretion of pro-inflammatory cytokine upon binding of the TCR to the antigen by the cell is increased between about 10% to about 500% as compared to secretion by a cell that does not have the exogenous CD8. In certain embodiments, LCK affinity of the cell is increased between about 10% to about 500% as compared to LCK affinity of a cell that does not have the exogenous CD8. In certain embodiments, persistence of the cell is increased between about 10% to about 500% as compared to persistence of a cell that does not have the exogenous CD8. In certain embodiments, tumor infiltration ability of the cell is increased between about 10% to about

500% as compared to tumor infiltration ability of a cell that does not have the exogenous CD8.

In certain embodiments, wherein the exogenous TCR is a CD8-dependent TCR. In certain embodiments, the exogenous TCR is a CD8-independent TCR. In certain embodiments, the exogenous CD8 is encoded by a CD8 Construct 1, a CD8 Construct 2, a CD8 Construct 3, or a CD8 Construct 4. In certain embodiments, the exogenous CD8 comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, and aCD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain. In certain embodiments, the exogenous CD8 comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

In certain embodiments, the presently disclosed subject matter provides a method of modifying a cell, the method comprising introducing into the cell a homologous recombination (HR) template nucleic acid sequence, wherein the HR template comprises first and second homology arms homologous to first and second target nucleic acid sequences, a TCR gene sequence positioned between the first and second homology arms, a CD8 gene sequence positioned between the first and the second homology arms, and recombining the HR template nucleic acid into an endogenous locus of the cell. In certain embodiments, the HR template comprises a first 2A-coding sequence positioned upstream of the CD8 gene sequence, a second 2A-coding sequence positioned downstream of the

CD8 gene sequence and upstream of the TCR gene sequence, and a third 2A-coding sequence positioned downstream of the TCR gene sequence; wherein the first, second, and third 2A-coding sequences code for the same amino acid sequence and are codon-diverged relative to each other. In certain embodiments, the HR template comprises a sequence coding for the amino acid sequence Gly Ser Gly positioned immediately upstream of the first, second, and/or third 2A-coding sequences. In certain embodiments, the HR template further comprises a sequence coding for a Furin cleavage site positioned upstream of the first, second, and/or third 2A-coding sequences. In certain embodiments, the HR template further comprises a sequence encoding a signal sequence positioned immediately upstream of the TCR gene sequence and/or the CD8 gene sequence.

In certain embodiments, the HR template comprises a second TCR sequence positioned between the third 2A-coding sequence and the second homology arm. In certain embodiments, the HR template comprises a sequence encoding a first signal sequence positioned immediately upstream the first TCR gene sequence, and a sequence encoding a second signal sequence positioned immediately upstream of the second TCR gene sequence. In certain embodiments, the HR template comprises a second CD8 gene sequence positioned between the first CD8 gene sequence and the second 2A-coding sequence. In certain embodiments, a 2A coding sequence is positioned between the first and second CD8 gene sequence. In certain embodiments, a sequence coding for the amino acid sequence Gly Ser Gly is positioned between the first and second CD8 gene sequences. In certain embodiments, a sequence coding for a Furin cleavage site is positioned between the first and second CD8 gene sequences.

In certain embodiments, the CD8 gene sequence comprises a sequence encoding an extracellular domain, a sequence encoding an intracellular domain, a sequence encoding an intracellular domain, fragments thereof, or combinations thereof. In certain embodiments, the sequence encoding an extracellular domain comprises a sequence encoding a CD8α extracellular domain or a CD8β extracellular domain. In certain embodiments, the sequence encoding a transmembrane domain comprises a sequence encoding a CD8α transmembrane domain or a CD8β transmembrane domain. In certain embodiments, the sequence encoding an intracellular domain comprises a sequence encoding a CD8α intracellular domain or a CD8β intracellular domain. In certain embodiments, the sequence encoding an intracellular domain comprises a sequence encoding a CD4 intracellular domain.

In certain embodiments, the CD8 gene sequence comprises a sequence encoding a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain. In certain embodiments, the CD8 gene sequence comprises a sequence encoding a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain. In certain embodiments, the CD8 gene sequence comprises a sequence encoding a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain. In certain embodiments, the CD8 gene sequence comprises a sequence encoding a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

In certain embodiments, the HR template comprises a sequence encoding a first signal sequence positioned immediately upstream the first CD8 gene sequence, and a sequence encoding a second signal sequence positioned immediately upstream of the second CD8 gene sequence. In certain embodiments, the signal sequence is a CD8 signal sequence, a human growth hormone signal sequence, fragments thereof, or combinations thereof. In certain embodiments, the first and second homology arms of the HR template are each from about 300 bases to about 2,000 bases in length. In certain embodiments, the first and second homology arms of the HR template are each from about 600 bases to about 2,000 bases in length.

In certain embodiments, the exogenous TCR is a patient derived TCR. In certain embodiments, the exogenous TCR comprises a signal sequence, a first and second 2A sequence, and a TCR polypeptide sequence. In certain embodiments, the exogenous TCR recognizes a cancer antigen. In certain embodiments, the cancer antigen is a neoantigen. In certain embodiments, the cancer antigen is a patient specific antigen. In certain embodiments, the HR template is non-viral. In certain embodiments, the HR template is a circular DNA. In certain embodiments, the HR template is a linear DNA. In certain embodiments, the introducing occurs via electroporation.

In certain embodiments, the recombining comprises cleavage of the endogenous locus by a nuclease, and recombination of the HR template nucleic acid sequence into the endogenous locus by homology directed repair. In certain embodiments, the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof. In certain embodiments, the nuclease further comprises a gRNA.

In certain embodiments, the method further comprises culturing the cell. In certain embodiments, the culturing is conducted in the presence of at least one cytokine. In certain embodiments, the culturing is conducted in the presence of IL2, IL7, IL15, or any

combination thereof. In certain embodiments, the culturing is conducted in the presence of IL7 and IL15. In certain embodiments, the method comprises a gene modification to enhance cell persistence and/or enhances memory cell differentiation.

In certain embodiments, the cell is a primary cell. In certain embodiments, the cell is a patient-derived cell. In certain embodiments, the cell is a lymphocyte. In certain embodiments, the cell is a T cell. In certain embodiments, the cell is a young T cell. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+. In certain embodiments, the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

In certain embodiments, killing activity of the cell is increased between about 10% to about 500% as compared to killing activity of a cell that does not have the CD8 gene sequence. In certain embodiments, proliferation of the cell upon binding of the TCR to the antigen is increased between about 10% to about 500% as compared to proliferation of a cell that does not have the CD8 gene sequence. In certain embodiments, secretion of pro-inflammatory cytokine upon binding of the TCR to the antigen by the cell is increased between about 10% to about 500% as compared to secretion by a cell that does not have the CD8 gene sequence. In certain embodiments, LCK affinity of the cell is increased between about 10% to about 500% as compared to LCK affinity of a cell that does not have the CD8 gene sequence. The method of any one of claims 43-95, wherein persistence of the cell is increased between about 10% to about 500% as compared to persistence of a cell that does not have the CD8 gene sequence. In certain embodiments, tumor infiltration ability of the cell is increased between about 10% to about 500% as compared to tumor infiltration ability of a cell that does not have the CD8 gene sequence.

In certain embodiments, the TCR gene encodes a CD8-dependent TCR. In certain embodiments, the TCR gene encodes a CD8-independent TCR. In certain embodiments, the CD8 gene sequence is encoded by a CD8 Construct 1, a CD8 Construct 2, a CD8 Construct 3, or a CD8 Construct 4. In certain embodiments, wherein the CD8 gene sequence comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or a CD8α extracellular domain, CD8α transmembrane domain, CD4 intracellular domain. In certain embodiments, the

CD8 gene sequence comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

In certain embodiments, the presently disclosed subject matter provides a cell modified by any of the methods disclosed herein.

In certain embodiments, the presently disclosed subject matter provides a composition comprising an effective amount of a cell disclosed herein. In certain embodiments, the composition is a pharmaceutical composition that further comprises a pharmaceutically acceptable excipient. In certain embodiments, the composition is administered to a patient in need thereof for the treatment of cancer. In certain embodiments, the composition comprises a cryopreservation agent. In certain embodiments, the composition comprises serum albumin. In certain embodiments, the composition comprises Plasma-Lyte A, HSA, and CryoStor CS10.

In certain embodiments, the presently disclosed subject matter provides a method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell or a composition disclosed herein.

In certain embodiments, prior to administering the therapeutically effective amount of cells disclosed herein, a non-myeloablative lymphodepletion regimen is administered to the subject. In certain embodiments, the cancer is a solid tumor. In certain embodiments, the cancer is a liquid tumor. In certain embodiments, the solid tumor is selected from the group consisting of melanoma, thoracic cancer, lung cancer, ovarian cancer, breast cancer, pancreatic cancer, head and neck cancer, prostate cancer, gynecological cancer, central nervous system cancer, cutaneous cancer, HPV+ cancer, esophageal cancer, thyroid cancer, gastric cancer, hepatocellular cancer,

cholangiocarcinomas, renal cell cancers, testicular cancer, sarcomas, and colorectal cancer. In certain embodiments, the liquid tumor is selected from the group consisting of follicular lymphoma, leukemia, and multiple myeloma.

In certain embodiments, the presently disclosed subject matter provides a kit comprising a cell disclosed herein, reagents for performing a method disclosed herein, or a composition disclosed herein. In certain embodiments, the kit further comprises written instructions for treating a cancer.

In certain embodiments, the presently disclosed subject matter provides a cell, comprising: an exogenous T cell receptor (TCR); and an exogenous CD8, comprising: a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

In certain embodiments, the presently disclosed subject matter provides a cell, comprising: an exogenous T cell receptor (TCR); and an exogenous CD8, comprising: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

In certain embodiments, the presently disclosed subject matter provides a method of modifying a cell, the method comprising: introducing into the cell a homologous recombination (HR) template nucleic acid sequence, wherein the HR template comprises: first and second homology arms homologous to first and second target nucleic acid sequences; a TCR gene sequence positioned between the first and second homology arms;

a CD8 gene sequence positioned between the first and the second homology arms; and recombining the HR template nucleic acid into an endogenous locus of the cell, wherein the CD8 gene sequence comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

In certain embodiments, the presently disclosed subject matter provides a method of modifying a cell, the method comprising: introducing into the cell a homologous recombination (HR) template nucleic acid sequence, wherein the HR template comprises: first and second homology arms homologous to first and second target nucleic acid sequences; a TCR gene sequence positioned between the first and second homology arms; a CD8 gene sequence positioned between the first and the second homology arms; and recombining the HR template nucleic acid into an endogenous locus of the cell, wherein the CD8 gene sequence comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

In certain embodiments, the presently disclosed subject matter provides a composition comprising a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β

intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

In certain embodiments, the presently disclosed subject matter provides a composition comprising a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

In certain embodiments, the presently disclosed subject matter provides a method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

In certain embodiments, the presently disclosed subject matter provides a method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO:

142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C. **Figures 1A-1C** show an example of a NeoE TCR cassette and gene editing methods that can be used to make NeoTCR Products. **Figure 1A** shows a schematic representing the general targeting strategy used for integrating neoantigen-specific TCR constructs (neoTCRs) into the TCRα locus. **Figures 1B** and **1C** show a neoantigen-specific TCR construct design used for integrating a NeoTCR into the TCRα locus wherein the cassette is shown with signal sequences ("SS"), protease cleavage sites ("P"), and 2A peptides ("2A"). **Figure 1B** shows a target TCRα locus (endogenous TRAC, top panel) and its CRISPR Cas9 target site (horizontal stripes, cleavage site designated by the arrow), and the circular plasmid HR template (bottom panel) with the polynucleotide encoding the neoTCR, which is located between left and right homology arms ("LHA" and "RHA" respectively) prior to integration. **Figure 1C** shows the integrated neoTCR in the TCRα locus (top panel), the transcribed and spliced neoTCR mRNA (middle panel), and translation and processing of the expressed neoTCR (bottom panel).

Figures 2A-2D. **Figures 2A-2D** show the circular plasmids used to encode CD8 constructs 1, 2, 3, and 4. **Figure 2A** shows CD8 Construct 1 used to produce the CD8 Product 1. **Figure 2B** shows CD8 Construct 2 used to produce the CD8 Product 2. **Figure 2C** shows CD8 Construct 3 used to produce the CD8 Product 3. **Figure 2D** shows CD8 Construct 4 used to produce the CD8 Product 4. As shown in **Figures 2A-2D**, SS stands for a signal sequence. As described in Figures **6**, **7**, **8**, and **9**, the SS may be HGH; however other signal sequences may be used as needed for appropriate trafficking. As shown in

Figures 2A-2D, P stands for a protease cleavage site. As described in Figures **6**, 7, **8**, and **9**, the P may be Furin; however other protease cleavage sites may be used as appropriate to provide the cleavage action described herein. As shown in **Figures 2A-2D**, 2A stands for the 2A peptide. As described in Figures **6**, 7, **8**, and **9**, the 2A may be the P2A peptide; however, other 2A peptides may be used.

Figure 3. **Figures 3A-3D** show the transcription/splicing and translation processing of each of CD8 Constructs 1, 2, 3, and 4 to yield CD8 Products 1, 2, 3, and 4. As shown in **Figures 3A-3D**, SS stands for a signal sequence. As described in Figures **6**, 7, **8**, and **9**, the SS may be HGH; however other signal sequences may be used as needed for appropriate trafficking. As shown in **Figures 3A-3D**, P stands for a protease cleavage site. As described in Figures **6**, 7, **8**, and **9**, the P may be Furin; however other protease cleavage sites may be used as appropriate to provide the cleavage action described herein. As shown in **Figures 3A-3D**, 2A stands for the 2A peptide. As described in Figures **6**, 7, **8**, and **9**, the 2A may be the P2A peptide; however, other 2A peptides may be used.

Figures 4A and **4B**. **Figure 4A** shows translated products of CD8 Product 1 and CD8 Product 2. **Figure 4B** shows the translated products of CD8 Product 3 and CD8 Product 4.

Figure 5. **Figure 5** provides an exemplary DNA sequence of the NeoTCR construct described in **Figures 1A-1C**. Conservative substitutions of nucleic acids can be used throughout to result in the same translated product. Furthermore, where amino acids can be substituted without a change in function, substitutions of the nucleic acids provided in **Figure 5** can also be used to achieve the substituted amino acids conferring a substantially similar or identical function of the translated proteins.

Figure 6. **Figure 6** provides an exemplary DNA sequence of the CD8 Construct 1 (and translated CD8 Product 1) described in **Figures 2A**, **3A**, and **4A**. Conservative substitutions of nucleic acids can be used throughout to result in the same translated product. Furthermore, where amino acids can be substituted without a change in function, substitutions of the nucleic acids provided in **Figure 6** can also be used to achieve the substituted amino acids conferring a substantially similar or identical function of the translated proteins.

Figure 7. **Figure** 7 provides an exemplary DNA sequence of the CD8 Construct 2 (and translated CD8 Product 2) described in **Figures 2B**, **3B**, and **4A**. Conservative substitutions of nucleic acids can be used throughout to result in the same translated product. Furthermore, where amino acids can be substituted without a change in function,

substitutions of the nucleic acids provided in **Figure 7** can also be used to achieve the substituted amino acids conferring a substantially similar or identical function of the translated proteins.

Figure 8. **Figure 8** provides an exemplary DNA sequence of the CD8 Construct 3 (and translated CD8 Product 3) described in **Figures 2C**, **3C**, and **4B**. Conservative substitutions of nucleic acids can be used throughout to result in the same translated product. Furthermore, where amino acids can be substituted without a change in function, substitutions of the nucleic acids provided in **Figure 8** can also be used to achieve the substituted amino acids conferring a substantially similar or identical function of the translated proteins.

Figure 9. **Figure 9** provides an exemplary DNA sequence of the CD8 Construct 4 (and translated CD8 Product 4) described in **Figures 2D**, **3D**, and **4B**. Conservative substitutions of nucleic acids can be used throughout to result in the same translated product. Furthermore, where amino acids can be substituted without a change in function, substitutions of the nucleic acids provided in **Figure 9** can also be used to achieve the substituted amino acids conferring a substantially similar or identical function of the translated proteins.

Figure 10. **Figure 10** presents a visual depiction of CD8 Products 1, 2, 3, and 4 along with the predicted LCK activity of each of CD8 Products 1, 2, 3, and 4.

Figures 11A-11D. **Figure 11A** provides an exemplary expression construct of CD8 Product 1. **Figure 11B** provides an exemplary expression construct of CD8 Product 2. **Figure 11C** provides an exemplary expression construct of CD8 Product 3. **Figure 11D** provides an exemplary expression construct of CD8 Product 4.

Figure 12. **Figure 12** diagrams the design of an Incucyte experiment to show the killing ability of the CD8 Products.

Figures 13A and **13B**. **Figures 13A** and **13B** show the increased tumor killing ability of CD8 Product 4 compared to a NeoTCR Product with the same NeoTCR as the CD8 Product 4 in a population of CD4 T cells. Specifically, both the CD8 Product 4 and the NeoTCR Product express TCR097. However, the CD8 Product 4 also expresses CD8 Construct 4 which comprises the extracellular domain of CD8α and the intracellular domain of CD4. The effector:target cell ratio (E:T Ration) was 1:1 (**Figure 13A**) or 2:1 (**Figure 13B**) and each were normalized for gene editing per cell line. The SW620 COX6C R20Q heterozygous tumor cells used in this experiment was a cell line that expresses the cognate antigen for TCR097 (a CD8 dependent TCR). This shows that the

low affinity binding of TCR097 can be saved by co-expressing the extracellular domain of CD8α and the intracellular domain of CD4.

Figure 14. **Figure 14** shows the increased tumor killing ability of CD8 Product 4 compared to a NeoTCR Product with the same NeoTCR as the CD8 Product 4 in a population of CD4 T cells. Specifically, both the CD8 Product 4 and the NeoTCR Product express TCR097. However, the CD8 Product 4 also expresses CD8 Construct 4 which comprises the extracellular domain of CD8a and the intracellular domain of CD4. The effector:target cell ratio (E:T Ration) was 1:1, 1:2, or 1:4 and each were normalized for gene editing per cell line. The SW620 COX6C R20Q homozygous tumor cells used in this experiment was a cell line that expresses the cognate antigen for TCR097. An increased killing can be seen in this experiment compared to that shown in **Figures 13A** and **13B** because the cell line is homozygous for the cognate antigen expression; thus, even though TCR97 is a low affinity TCR, the increased amount of cognate antigen overcame the low affinity limitations.

Figures 15A and **15B**. **Figures 15A** and **15B** provide exemplary control experiments showing that there is an increased tumor killing ability of CD8 Product 4 compared to a NeoTCR Product with the same NeoTCR as the CD8 Product 4 in a population of CD4 T cells (top graphs in **Figures 15A** and **15B**) or CD8 T cells (bottom graphs in **Figures 15A** and **15B**) and that CD8 Product 4 is more effective at tumor killing than simply expressing the NeoTCR construct and CD8 Product 4 in CD8 T cells. **Figure 15A** shows an E:T ration of 2:1 and **Figure 15B** shows an E:T ratio of 1:1.

Figure 16. **Figure 16** shows the surface expression of CD8 Construct 4. Peak #1 is a NeoTCR Product in CD4+/CD8- T cells (expressing TCR089). Peak #2 is a CD8 Product 4 in CD8+/CD4+ T cells. Peak #3 is a NeoTCR Product in CD8+/CD4- T cells. As shown CD8 Construct 4 exhibited proper and comparable surface expression as the NeoTCR Products. Similar results were achieved with the other CD8 Constructs (data not shown).

Figures 17A and 17B. **Figures 17A** and **17B** shows that CD8α expression boosts CD4 T cell sensitivity while maintaining specificity. **Figure 17A** shows that the expression of CD8 Constructs 1-4 increases the sensitivity of the T cells and does not change the specificity of the CD8 Product to the NeoTCR. As shown, co-expression of the CD8 Constructs decreases the EC50 which shows the increased sensitivity to the NeoTCR. **Figure 17B** shows that the CD8 Products 1-4 are specific for the cognate antigen to NeoTCR 097 because there is only INFγ production by the CD8 Cells of the

CD8 Products 1-4 in the presence of cognate antigen (i.e., no INFγ production by the CD8 Cells of the CD8 Products 1-4 in the presence of mismatched antigen). The same experiment as shown in **Figures 17A** and **17B** were performed with CD107a instead of INFγ and the same increased sensitivity with maintained specificity to the NeoTCR was shown (data not shown) to confirm the results with INFγ.

Figure 18. **Figure 18** shows that CD8α expression boosts CD4 T cell sensitivity among CD8-independent NeoTCRs. The figure shows data from the CD8-independent NeoTCR089. This shows that even when a NeoTCR is CD8-independent, there is an increase in sensitivity of CD8 Product 4. This was a surprising result to find that sensitivity can be increased even for CD8-independent TCRs. Similar results were achieved with CD8 Products 1-3 (data not shown). Thus, CD8-dependent (e.g., NeoTCR097) and CD8-independent (e.g., NeoTCR089) exhibit increased sensitivity compared to NeoTCR Products expressing the same NeoTCRs.

DETAILED DESCRIPTION

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art. The following references provide one of skill with a general definition of many of the terms used in the presently disclosed subject matter: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments. The terms “comprises” and “comprising” are intended to have the broad meaning ascribed to them in U.S. Patent Law and can mean “includes”, “including” and the like.

As used herein, the term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the

measurement system. For example, “about” can mean within 3 or more than 3 standard deviations, per the practice in the art. Alternatively, “about” can mean a range of up to 20%, e.g., up to 10%, up to 5%, or up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, e.g., within 5-fold or within 2-fold, of a value.

The term "antibody" as used herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific and tri-specific antibodies), and antibody fragments (e.g., bis-Fabs) so long as they exhibit the desired antigen-binding activity. "Antibody Fragment" as used herein refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to bis-Fabs; Fv; Fab; Fab, Fab'-SH; $F(ab')_2$; diabodies; linear antibodies; single-chain antibody molecules (e.g., scFv); and multispecific antibodies formed from antibody fragments.

The terms “Cancer” and “Tumor” are used interchangeably herein. As used herein, the terms “Cancer” or “Tumor” refer to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms are further used to refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Cancer can affect a variety of cell types, tissues, or organs, including but not limited to an organ selected from the group consisting of bladder, bone, brain, breast, cartilage, glia, esophagus, fallopian tube, gallbladder, heart, intestines, kidney, liver, lung, lymph node, nervous tissue, ovaries, pancreas, prostate, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, and vagina, or a tissue or cell type thereof. Cancer includes cancers, such as sarcomas, carcinomas, or plasmacytomas (malignant tumor of the plasma cells). Examples of cancer include, but are not limited to, those described herein. The terms “Cancer” or “Tumor” and “Proliferative Disorder” are not mutually exclusive as used herein.

“CD8” is a cell surface glycoprotein found on most cytotoxic T lymphocytes that mediates efficient cell-cell interactions within the immune system. The CD8 antigen acts as a coreceptor with the T-cell receptor on the T lymphocyte to recognize antigens displayed by an antigen presenting cell in the context of class I MHC molecules.

“CD8 Cells” as used herein means one or more cells precision engineered to express one or more NeoTCRs and a CD8 Construct.

"CD8 Construct" as used herein means any one of a CD8 Construct 1, a CD8 Construct 2, a CD8 Construct 3, or a CD8 Construct 4.

"CD8 Product" as used herein means a product comprising CD8 Cells.

"CD8 Construct 1" and "CD8 Product 1" refer to a construct that comprises a NeoTCR and CD8α (CD8α extracellular domain, CD8α transmembrane domain, and CD8α intracellular domain) and the resulting product that comprises an expressed NeoTCR and CD8α. Non-limiting examples of a CD8 Product 1 is provided in **Figures 2A**, **3A**, and **6**. A non-limiting example of a CD8 Product 1 is provided in **Figure 4A**.

"CD8 Construct 2" and "CD8 Product 2" refer to a construct that comprises a NeoTCR, CD8α (CD8α extracellular domain, CD8α transmembrane domain, and CD8α intracellular domain), and CD8β (CD8β extracellular domain, CD8β transmembrane domain, and CD8β intracellular domain) and the resulting product that comprises an expressed NeoTCR, CD8α, and CD8β. Non-limiting examples of a CD8 Product 2 is provided in **Figures 2B**, **3B**, and 7. A non-limiting example of a CD8 Product 1 is provided in **Figure 4A**.

"CD8 Construct 3" and "CD8 Product 3" refer to a construct that comprises a NeoTCR, CD8α extracellular domain, CD8α transmembrane domain, and CD8β intracellular domain and the resulting product that comprises an expressed NeoTCR, CD8α extracellular domain, CD8α transmembrane domain, and CD8β intracellular domain. Non-limiting examples of a CD8 Product 3 is provided in **Figures 2C**, **3C**, and **8**. A non-limiting example of a CD8 Product 1 is provided in **Figure 4B**.

"CD8 Construct 4" and "CD8 Product 4" refer to a construct that comprises a NeoTCR, CD8α extracellular domain, CD8α transmembrane domain, and CD4 intracellular domain) and the resulting product that comprises an expressed NeoTCR, CD8α extracellular domain, CD8α transmembrane domain, and CD4 intracellular domain. Non-limiting examples of a CD8 Product 4 is provided in **Figures 2D**, **3D**, and **9**. A non-limiting example of a CD8 Product 1 is provided in **Figure 4B**.

A "conservative substitution" or a "conservative amino acid," refers to the substitution of an amino acid with a chemically or functionally similar amino acid. Conservative substitution tables providing similar amino acids are well known in the art.

In certain embodiments, acidic amino acids D and E are conservative substitutions for one another; basic amino acids K, R, and H are conservative substitutions for one another; hydrophilic uncharged amino acids S,T ,N. and Q are conservative substitutions for one another; aliphatic uncharged amino acids G, A, V, L, and I are conservative

substitutions for one another; non-polar uncharged amino acids C, M, and P are conservative substitutions for one another; aromatic amino acids F, Y, and W are conservative substitutions for one another; A, S, and T are conservative substitutions for one another; D and E are conservative substitutions for one another; N and Q are conservative substitutions for one another; R and K are conservative substitutions for one another; I, L, and M are conservative substitutions for one another; F, Y, and W are conservative substitutions for one another; A and G are conservative substitutions for one another; D and E are conservative substitutions for one another; N and Q are conservative substitutions for one another; R, K and H are conservative substitutions for one another; I, L, M, and V are conservative substitutions for one another; F, Y and W are conservative substitutions for one another; S and T are conservative substitutions for one another; and C and M are conservative substitutions for one another.

Additional conservative substitutions may be found, for example, in Creighton, *Proteins: Structures and Molecular Properties* 2nd ed. (1993) W. H. Freeman & Co., New York, NY.

"Treat," "Treatment," and "treating" are used interchangeably and as used herein mean obtaining beneficial or desired results including clinical results. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, the NeoTCR Product of the invention is used to delay development of a proliferative disorder (e.g., cancer) or to slow the progression of such disease.

"Dextramer" as used herein means a multimerized neoepitope-HLA complex that specifically binds to its cognate NeoTCR.

As used herein, the terms "neoantigen", "neoepitope" or "neoE" refer to a newly formed antigenic determinant that arises, e.g., from a somatic mutation(s) and is recognized as "non-self." A mutation giving rise to a "neoantigen", "neoepitope" or "neoE" can include a frameshift or non-frameshift indel, missense or nonsense substitution, splice site alteration (e.g., alternatively spliced transcripts), genomic rearrangement or gene fusion, any genomic or expression alterations, or any post-translational modifications.

"NeoTCR", "NeoE TCR" and "exogenous TCR" as used herein mean a neoepitope-specific T cell receptor that is introduced into a T cell, e.g., by gene editing

methods. As used herein, the term "TCR gene sequence" refers to a NeoTCR gene sequence.

"NeoTCR cells" as used herein means one or more cells precision engineered to express one or more NeoTCRs. In certain embodiments, the cells are T cells. In certain embodiments, the T cells are CD8+ and/or CD4+ T cells. In certain embodiments, the CD8+ and/or CD4+ T cells are autologous cells from the patient for whom a NeoTCR Product will be administered. The terms "NeoTCR cells" and "NeoTCR-P1 T cells" and "NeoTCR-P1 cells" are used interchangeably herein.

"NeoTCR Product" as used herein means a pharmaceutical formulation comprising one or more NeoTCR cells. NeoTCR Product consists of autologous precision genome-engineered CD8+ and CD4+ T cells. Using a targeted DNA-mediated *non-viral* precision genome engineering approach, expression of the endogenous TCR is eliminated and replaced by a patient-specific NeoTCR isolated from peripheral CD8+ T cells targeting the tumor-exclusive neoepitope. In certain embodiments, the resulting engineered CD8+ or CD4+ T cells express NeoTCRs on their surface of native sequence, native expression levels, and native TCR function. The sequences of the NeoTCR external binding domain and cytoplasmic signaling domains are unmodified from the TCR isolated from native CD8+ T cells. Regulation of the NeoTCR gene expression is driven by the native endogenous TCR promoter positioned upstream of where the NeoTCR gene cassette is integrated into the genome. Through this approach, native levels of NeoTCR expression are observed in unstimulated and antigen-activated T cell states.

The NeoTCR Product manufactured for each patient represents a defined dose of autologous CD8+ and/or CD4+ T cells that are precision genome engineered to express a single neoE-specific TCR cloned from neoE-specific CD8+ T cells individually isolated from the peripheral blood of that same patient.

"NeoTCR Viral Product" as used herein has the same definition of NeoTCR Product except that the genome engineering is performed using viral mediated methods.

"Pharmaceutical Formulation" refers to 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. For clarity, DMSO at quantities used in a NeoTCR Product is not considered unacceptably toxic.

A "subject," "patient," or an "individual" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo,

sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

"TCR" as used herein means T cell receptor.

The term "tumor antigen" as used herein refers to an antigen (e.g., a polypeptide) that is uniquely or differentially expressed on a tumor cell compared to a normal or non-neoplastic cell. In certain embodiments, a tumor antigen includes any polypeptide expressed by a tumor that is capable of activating or inducing an immune response via an antigen-recognizing receptor or capable of suppressing an immune response via receptor-ligand binding.

"2A" and "2A peptide" are used interchangeably herein and mean a class of 18-22 amino acid long, viral, self-cleaving peptides that are able to mediate cleavage of peptides during translation in eukaryotic cells.

Four well-known members of the 2A peptide class are T2A, P2A, E2A, and F2A. The T2A peptide was first identified in the Thosea asigna virus 2A. The P2A peptide was first identified in the porcine teschovirus-1 2A. The E2A peptide was first identified in the equine rhinitis A virus. The F2A peptide was first identified in the foot-and-mouth disease virus.

The self-cleaving mechanism of the 2A peptides is a result of ribosome skipping the formation of a glycyl-prolyl peptide bond at the C-terminus of the 2A. Specifically, the 2A peptides have a C-terminal conserved sequence that is necessary for the creation of steric hindrance and ribosome skipping. The ribosome skipping can result in one of three options: 1) successful skipping and recommencement of translation resulting in two cleaved proteins (the upstream of the 2A protein which is attached to the complete 2A peptide except for the C-terminal proline and the downstream of the 2A protein which is attached to one proline at the N-terminal; 2) successful skipping but ribosome fall-off that results in discontinued translation and only the protein upstream of the 2A; or 3) unsuccessful skipping and continued translation (i.e., a fusion protein).

The term "endogenous" as used herein refers to a nucleic acid molecule or polypeptide that is normally expressed in a cell or tissue.

The term "exogenous" as used herein refers to a nucleic acid molecule or polypeptide that is not endogenously present in a cell. The term "exogenous" would therefore encompass any recombinant nucleic acid molecule or polypeptide expressed in a cell, such as foreign, heterologous, and over-expressed nucleic acid molecules and polypeptides. By "exogenous" nucleic acid is meant a nucleic acid not present in a native

wild-type cell; for example, an exogenous nucleic acid may vary from an endogenous counterpart by sequence, by position/location, or both. For clarity, an exogenous nucleic acid may have the same or different sequence relative to its native endogenous counterpart; it may be introduced by genetic engineering into the cell itself or a progenitor thereof, and may optionally be linked to alternative control sequences, such as a non-native promoter or secretory sequence.

"Young" or "Younger" or "Young T cell" as it relates to T cells means memory stem cells (T_{MSC}) and central memory cells (T_{CM}). These cells have T cell proliferation upon specific activation and are competent for multiple cell divisions. They also have the ability to engraft after re-infusion, to rapidly differentiate into effector T cells upon exposure to their cognate antigen and target and kill tumor cells, as well as to persist for ongoing cancer surveillance and control.

NeoTCR PRODUCTS

In some embodiments, using the gene editing technology and neoTCR isolation technology described in PCT/US2020/17887 and PCT/US2019/025415, which are incorporated herein in their entireties, NeoTCRs are cloned in autologous CD8+ and CD4+ T cells from the same patient with cancer by precision genome engineered (using a DNA-mediated (non-viral) method as described in **Figures 1A-1C**) to express the neoTCR. In other words, the NeoTCRs that are tumor specific are identified in cancer patients, such NeoTCRs are then cloned, and then the cloned NeoTCRs are inserted into the cancer patient's T cells. NeoTCR expressing T cells are then expanded in a manner that preserves a "young" T cell phenotypes, resulting in a NeoTCR-P1 product (i.e., a NeoTCR Product) in which the majority of the T cells exhibit T memory stem cell and T central memory phenotypes.

These 'young' or 'younger' or less-differentiated T cell phenotypes are described to confer improved engraftment potential and prolonged persistence post-infusion. Thus, the administration of NeoTCR Product, consisting significantly of 'young' T cell phenotypes, has the potential to benefit patients with cancer, through improved engraftment potential, prolonged persistence post-infusion, and rapid differentiation into effector T cells to eradicate tumor cells throughout the body.

Ex vivo mechanism-of-action studies were also performed with NeoTCR Product manufactured with T cells from patients with cancer. Comparable gene editing efficiencies

and functional activities, as measured by antigen-specificity of T cell killing activity, proliferation, and cytokine production, were observed demonstrating that the manufacturing process described herein is successful in generating products with T cells from patients with cancer as starting material.

In certain embodiments, the NeoTCR Product manufacturing process involves electroporation of dual ribonucleoprotein species of CRISPR-Cas9 nucleases bound to guide RNA sequences, with each species targeting the genomic TCRα and the genomic TCRβ loci. The specificity of targeting Cas9 nucleases to each genomic locus has been previously described in the literature as being highly specific. Comprehensive testing of the NeoTCR Product was performed *in vitro* and *in silico* analyses to survey possible off-target genomic cleavage sites, using COSMID and GUIDE-seq, respectively. Multiple NeoTCR Product or comparable cell products from healthy donors were assessed for cleavage of the candidate off-target sites by deep sequencing, supporting the published evidence that the selected nucleases are highly specific.

Further aspects of the precision genome engineering process have been assessed for safety. No evidence of genomic instability following precision genome engineering was found in assessing multiple NeoTCR Products by targeted locus amplification (TLA) or standard FISH cytogenetics. No off-target integration anywhere into the genome of the NeoTCR sequence was detected. No evidence of residual Cas9 was found in the cell product.

The comprehensive assessment of the NeoTCR Product and precision genome engineering process indicates that the NeoTCR Product will be well tolerated following infusion back to the patient.

The genome engineering approach described herein enables highly efficient generation of bespoke NeoTCR T cells (i.e., NeoTCR Products) for personalized adoptive cell therapy for patients with solid and liquid tumors. Furthermore, the engineering method is not restricted to the use in T cells and has also been applied successfully to other primary cell types, including natural killer and hematopoietic stem cells.

CD8 PRODUCTS

Coexpression of MHC class I–restricted neoTCRs and ectopic CD8 receptors in precision genome engineered CD4 T cells significantly potentiates antigen-specific effector functions.

Neoepitopes from tumor-exclusive mutations represent compelling targets for personalized neoE-specific autologous TCR-T cell therapies for patients with solid tumors. The imPACT Isolation Technology as described in PCT/US2020/17887, which is incorporated by reference in its entirety, is an ultra-sensitive and high-throughput process for capturing neoE-specific CD8 T cells from the blood of patients with solid cancers. Leveraging this technology, neoepitope-specific MHC class I-restricted TCRs ("MHC-I neoTCRs") were cloned from individually captured CD8 T cells. Using DNA-mediated (non-viral) gene editing as described in **Example 1**, fresh CD8 and CD4 T cells from the same patient with cancer were engineered to express the MHC-I neoTCR (concomitant with elimination of the endogenous TCR).

While naturally occurring MHC-I TCRs were presumed to require concurrent CD8 co-receptor help to stabilize peptide-MHC binding, higher affinity TCRs were able to drive CD8-independent target binding and T cell activation. CD4 T cells, when engineered with high affinity neoTCRs, were thus able to recognize peptide-MHC-I targets and trigger effector T cell functions. However, lower affinity TCRs were dependent on CD8 co-receptors to trigger T cell activation. By precision genome engineering CD8 co-receptor genes together with the neoTCR into CD4 T cells, MHC-I neoTCRs were made competent to trigger antigen-specific effector T cell function.

CD8 stabilizes TCR-pMHC interactions and synergizes with TCRs for avidity. CD8 also serves to enhance avidity from low affinity TCRs, while the intracellular domain of CD8α is critical for enhanced T cell activation. Expression of CD8 may enhance CD4 T cell responses, which may not respond to physiological concentrations of pMHC. Disruption of CD8 binding to MHC can convert catch-bond TCR-pMHC into slip-bonds, highlighting the importance of CD8-MHC interactions even for TCRs that bind pMHC independent of CD8.

CD8α has a lower affinity to LCK than CD8β, suggesting that either co-expression of both CD8α and CD8β or generation of a chimeric CD8α-CD8β molecule which contains the extracellular CD8α and intracellular domain of CD8β can improve effectiveness. The CD8 Constructs described herein are:

1. CD8α homodimer (CD8 Construct 1)
2. CD8α-P2A-CD8β (CD8 Construct 2)
3. CD8α with CD8β intracellular domain (CD8 Construct 3)
4. CD8α homodimer with CD4 intracellular domain (CD8 Construct 4)

In some embodiments, the NeoTCR Products described above include an additional modification to include the expression of CD8 Construct 1, CD8 Construct 2, CD8 Construct 3, or CD8 Construct 4 (each a CD8 Product). Specifically, using the gene editing technology and neoTCR isolation technology described in PCT/US2020/17887 and PCT/US2019/025415, which are incorporated herein in their entireties, NeoTCRs are cloned in autologous CD8+ and CD4+ T cells from the same patient with cancer by precision genome engineered (using a DNA-mediated (non-viral) method as described in **Figures 1A-1C**) to express the neoTCR.

Each of the CD8 Constructs, when expressed, result in CD8 Products. **Table 1** provides a description of each construct and product.

Table 1. CD8 Products and CD8 Constructs

CD8 Construct	CD8 Product	Expression components	Exemplary constructs	Exemplary products
CD8 Construct 1	CD8 Product 1	NeoTCR, CD8α extracellular domain, CD8α transmembrane domain, CD8α intracellular domain	**Figure 2A**, **Figure 6**	**Figure 3A**, **Figure 4A**
CD8 Construct 2	CD8 Product 1	NeoTCR, CD8α extracellular domain, CD8α transmembrane domain, CD8α intracellular domain, CD8β extracellular domain, CD8β transmembrane domain, CD8β intracellular domain	**Figure 2B**, **Figure 7**	**Figure 3B**, **Figure 4A**
CD8 Construct 3	CD8 Product 1	NeoTCR, CD8α extracellular domain, CD8α transmembrane domain, CD8β intracellular domain	**Figure 2C**, **Figure 8**	**Figure 3C**, Figure 4B
CD8 Construct 4	CD8 Product 1	NeoTCR, CD8α extracellular domain, CD8α transmembrane domain, CD4 intracellular domain	**Figure 2D**, **Figure 9**	**Figure 3D**, **Figure 4B**

In certain embodiments, the CD8 Product 1 comprises a NeoTCR and a CD8 homodimer. In certain embodiments, the CD8 Product 1 comprises the expression of a NeoTCR, a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain. In certain embodiments, the CD8 Product 1 further includes the expression of a CD8α signal peptide. In certain embodiments, the CD8 Product 1 comprises the translated elements presented in **Figure 3A**. In a non-limiting exemplary

embodiment, the CD8 Product 1 comprises a NeoTCR, a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142). In certain embodiments, sequence modifications of the CD8α signal peptide, CD8α extracellular domain, CD8α transmembrane domain, and CD8α intracellular domain can be made that conserve or substantially conserve function of each element. In certain embodiments, such sequence modifications are conservative substitutions of amino acids.

In a non-limiting embodiment, the CD8 Product 1 is manufactured from a CD8 Construct 1 provided in **Figure 6**. In certain embodiments, the sequence of CD8 Construct 1 provided in **Figure 6** can be modified in any number of ways so long as the translation of the CD8α signal peptide, CD8α extracellular domain, CD8α transmembrane domain, and CD8α intracellular domain leave each element with conserved function. In certain embodiments, the order of each element of the CD8 Construct 1 in **Figure 6** remains the same but the sequences of each individual element can be changed so long as the amino acids that the nucleic acid encodes remain the same or only comprise conservative substitutions. In certain embodiments, the order of each element of the CD8 Construct 1 in **Figure 6** remains the same but the sequences of each individual element can be changed so long as the function of the encoded proteins remains substantially unchanged.

In certain embodiments, the CD8 Product 2 comprises a NeoTCR, a CD8α, and aCD8β. In certain embodiments, the CD8α and CD8β are separated by a protease cleavage site and a 2A peptide in the CD8 Product 2 construct for expression. In certain embodiments, the CD8 Product 2 comprises the expression of a NeoTCR, a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain. In certain embodiments, the CD8 Product 2 further comprises the expression of a CD8α signal peptide. In certain embodiments, the CD8 Product further comprises the expression of a CD8β signal peptide. In certain embodiments, the CD8 Product further comprises the expression of a CD8α signal peptide and a CD8β signal peptide. In certain embodiments, the CD8 Product 2 comprises the translated elements presented in **Figure 3B**. In a non-limiting exemplary embodiment, the CD8 Product 2 comprises a NeoTCR, a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular

domain (SEQ ID NO: 147). In certain embodiments, sequence modifications of the CD8α signal peptide, CD8α extracellular domain, CD8α transmembrane domain, CD8α intracellular domain, CD8β extracellular domain, CD8β transmembrane domain, and CD8β intracellular domain can be made that conserve or substantially conserve function of each element. In certain embodiments, such sequence modifications are conservative substitutions of amino acids.

In a non-limiting embodiment, the CD8 Product 2 is manufactured from a CD8 Construct 2 provided in **Figure** 7. In certain embodiments, the sequence of CD8 Construct 2 provided in **Figure** 7 can be modified in any number of ways so long as the translation of the CD8α signal peptide, CD8α extracellular domain, CD8α transmembrane domain, CD8α intracellular domain, CD8β extracellular domain, CD8β transmembrane domain, and CD8β intracellular domain leave each element with conserved function. In certain embodiments, the order of each element of the CD8 Construct 2 in **Figure** 7 remains the same but the sequences of each individual element can be changed so long as the amino acids that the nucleic acid encodes remain the same or only comprise conservative substitutions. In certain embodiments, the order of each element of the CD8 Construct 2 in **Figure** 7 remains the same but the sequences of each individual element can be changed so long as the function of the encoded proteins remains substantially unchanged.

In certain embodiments, the CD8 Product 3 comprises a NeoTCR and a CD8α with CD8β intracellular domain. In certain embodiments, the CD8 Product 3 comprises the expression of a NeoTCR, a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain. In certain embodiments, the CD8 Product 3 further includes the expression of a CD8α signal peptide. In certain embodiments, the CD8 Product 3 comprises the translated elements presented in **Figure 3C**. In a non-limiting exemplary embodiment, the CD8 Product 3 comprises a NeoTCR, a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147). In certain embodiments, sequence modifications of the CD8α signal peptide, CD8α extracellular domain, CD8α transmembrane domain, and CD8β intracellular domain can be made that conserve or substantially conserve function of each element. In certain embodiments, such sequence modifications are conservative substitutions of amino acids.

In a non-limiting embodiment, the CD8 Product 3 is manufactured from a CD8 Construct 3 provided in **Figure 8**. In certain embodiments, the sequence of CD8 Construct 3 provided in **Figure 8** can be modified in any number of ways so long as the translation

of the CD8α signal peptide, CD8α extracellular domain, CD8α transmembrane domain, and CD8β intracellular domain leave each element with conserved function. In certain embodiments, the order of each element of the CD8 Construct 3 in **Figure 8** remains the same but the sequences of each individual element can be changed so long as the amino acids that the nucleic acid encodes remain the same or only comprise conservative substitutions. In certain embodiments, the order of each element of the CD8 Construct 3 in **Figure 8** remains the same but the sequences of each individual element can be changed so long as the function of the encoded proteins remains substantially unchanged.

In certain embodiments, the CD8 Product 4 comprises a NeoTCR and a CD8α homodimer with a CD4 intracellular domain. In certain embodiments, the CD8 Product 4 comprises the expression of a NeoTCR, a CD8α extracellular domain, aCD8α transmembrane domain, and a CD4 intracellular domain. In certain embodiments, the CD8 Product 4 further includes the expression of a CD8α signal peptide. In certain embodiments, the CD8 Product 4 comprises the translated elements presented in **Figure 3D**. In a non-limiting exemplary embodiment, the CD8 Product 4 comprises a NeoTCR, a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148). In certain embodiments, sequence modifications of the CD8α signal peptide, CD8α extracellular domain, CD8α transmembrane domain, and CD4 intracellular domain can be made that conserve or substantially conserve function of each element. In certain embodiments, such sequence modifications are conservative substitutions of amino acids.

In a non-limiting embodiment, the CD8 Product 4 is manufactured from a CD8 Construct 4 provided in **Figure 9**. In certain embodiments, the sequence of CD8 Construct 4 provided in **Figure 9** can be modified in any number of ways so long as the translation of the CD8α signal peptide, CD8α extracellular domain, CD8α transmembrane domain, and CD4 intracellular domain leave each element with conserved function. In certain embodiments, the order of each element of the CD8 Construct 4 in **Figure 9** remains the same but the sequences of each individual element can be changed so long as the amino acids that the nucleic acid encodes remain the same or only comprise conservative substitutions. In certain embodiments, the order of each element of the CD8 Construct 4 in **Figure 9** remains the same but the sequences of each individual element can be changed so long as the function of the encoded proteins remains substantially unchanged.

In certain embodiments, the CD8 Products comprise a TET2 knockout or TET2 knockdown. In certain embodiments, the cells of the CD8 Product are further engineered

to knockout the TET2 gene using non-viral methods. In certain embodiments, the cells of the CD8 Product are further engineered to knockout the TET2 gene using viral methods. In certain embodiments, the cells of the CD8 Product are further engineered to increase T cell persistence by knocking out, knocked down, or modifying the function of a gene associated with T cell persistence using non-viral methods. In certain embodiments, the cells of the CD8 Product are further engineered to increase T cell persistence by knocking out, knocking down, or modifying the function of a gene associated with T cell persistence using viral methods.

In some embodiments, the CD8 Products comprise cells that were engineered to express a NeoTCR and a CD8 Construct using viral methods (i.e., CD8 Viral Product). In certain embodiments, the cells of the CD8 Viral Product are further engineered to knockout the TET2 gene using non-viral methods. In certain embodiments, the cells of the CD8 Viral Product are further engineered to knockout the TET2 gene using viral methods. In certain embodiments, the cells of the CD8 Viral Product are further engineered to increase T cell persistence by knocking out, knocking down, or modifying the function of a gene associated with T cell persistence using non-viral methods. In certain embodiments, the cells of the CD8 Viral Product are further engineered to increase T cell persistence by knocking out, knocking down, or modifying the function of a gene associated with T cell persistence using viral methods.

In certain embodiments, the T cell persistence gene that is knocked out, knocked down, or with a modified function is a gene that confers downregulation of T cell activity. In certain embodiments, the gene that is knocked out, knocked down, or modified is a gene that downregulates T cell memory function. In certain embodiments, the gene that is knocked out, knocked down, or modified is a gene that decreases T-cell function, proliferation, and/or survival.

In certain embodiments, additional modifications to the CD8 Products and CD8 Cells thereof include modifications to increase tumor microenvironment resilience, increase T cell activity, increase tumor microenvironment homing/retention, increase T cell persistence, and increase ectopic effector functions of T cells. In certain embodiments, the tumor microenvironment resilience includes but is not limited to converting/counteracting negative environmental signals. In certain embodiments, TCR-mediated signal enhancements include but are not limited to increasing T cell activity. In certain embodiments, the tumor microenvironment homing/retention includes but is not limited to enhancing tumor infiltration. In certain embodiments, the functional T cell

persistence includes but is not limited to metabolic and transcriptional regulation. In certain embodiments, the ectopic effector functions include but is not limited to TCR-induced antibody, cytokine, or peptide secretion.

In certain embodiments, functional T cell persistence can be accomplished by genetic engineering as described herein, by co-administration of a pharmaceutical agent that improves the functional T cell persistence of a modified T cells (modified to incorporate at least a neoTCR as described herein), and by manufacturing and culture conditions of the modified T cells (modified to incorporate at least a neoTCR as described herein).

In certain embodiments, the TCR-induced antibodies used to improve ectopic effector functions are any one of the antibodies or functional fragments thereof described herein. In certain embodiments, the TCR-induced cytokines used to improve ectopic effector functions are naturally occurring cytokines, modified cytokines, fusion proteins of the cytokines, or any combination thereof. In certain embodiments, the TCR-induced cytokines are not cytokines but rather other co-factors or expression elements that induce endogenous cytokine production.

In certain embodiments, additional modifications to the CD8 Products and CD8 Cells thereof include modifications to knock in one or more additional genes and/or functional proteins. In certain embodiments, the genes and/or functional proteins knocked in include but are not limited c-Myb, dominant negative FAS, FAS truncations, FBXW7, CTP1A, OPA1, GLUT1, CA-STAT5A, dominant negative TGFβR, DNMT3a, dominant negative PD-1R, dominant negative PD-1 or PD-L1, or PD-L2, dominant negative SHIP-1 protein, integrins, chemokine receptors, cytokines, and interleukins. In certain embodiments, the dominant negative form of a gene is an antibody or functional fragment thereof that is an antagonist of the gene. For example, a dominant negative PD-1 can be an anti-PD-1 antibody or functional fragment thereof. In certain embodiments, any of the knock in genes and/or functional proteins is a functional fragment (including but not limited to truncations) of the gene and/or protein.

In certain embodiments, additional modifications to the CD8 Products and CD8 Cells thereof include modifications to knock out or knock down of one or more additional genes and/or functional proteins. In certain embodiments, the genes and/or functional proteins knocked out or knocked down include but are not limited to TET2, IFNGR1, RICTOR, NR4A1, DNMT3A, SUV39H1, PPP2RD, adenosine 2A receptor, PP2A3, and PP2A4.

In certain embodiments, instead of knocking in a gene for the expression of a functional protein, a different gene can be modulated through genetic engineering described herein that upregulates the expression of the gene that would otherwise be knocked in.

In certain embodiments, instead of knocking out or knocking out a gene, a different gene can be modulated through genetic engineering described herein that downregulates the expression of the gene that would otherwise be knocked out.

The CD8 Product manufacturing process involves electroporation of dual ribonucleoprotein species of CRISPR-Cas9 nucleases bound to guide RNA sequences, with each species targeting the genomic TCRα and the genomic TCRβ loci. The specificity of targeting Cas9 nucleases to each genomic locus has been previously described in the literature as being highly specific. Comprehensive testing of the CD8 Product was performed *in vitro* and *in silico* analyses to survey possible off-target genomic cleavage sites, using COSMID and GUIDE-seq, respectively. Multiple CD8 Product or comparable cell products from healthy donors were assessed for cleavage of the candidate off-target sites by deep sequencing, supporting the published evidence that the selected nucleases are highly specific.

In certain embodiments, the CD8 Products described herein can be T cells, NK cells, NKT cells, macrophages, hematopoietic stem cells (HSCs), cells derived from HSCs, or dendritic/antigen-presenting cells.

In certain embodiments, CD8 Cells are expanded in a manner that preserves a "young" T cell phenotypes, resulting in a CD8 Product in which the majority of the T cells exhibit T memory stem cell and T central memory phenotypes

These 'young' or 'younger' or less-differentiated T cell phenotypes are described to confer improved engraftment potential and prolonged persistence post-infusion. Thus, the administration of CD8 Product, consisting significantly of 'young' T cell phenotypes, has the potential to benefit patients with cancer, through improved engraftment potential, prolonged persistence post-infusion, and rapid differentiation into effector T cells to eradicate tumor cells throughout the body.

In certain embodiments, the CD8 Cells of the CD8 Products predominantly comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 25% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 30% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central

memory cells (Tcm). In certain embodiments, at least 35% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 40% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 45% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 50% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 55% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 60% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 65% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 70% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 75% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, greater than 75% of the CD8 Cells of the CD8 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). Tmsc are characterized as cells that are CD45RA+CD62L+, CD28+CD95+, and CCR7+CD27+. Tcm are characterized as cells that are CD45RO+CD62L+, CD28+CD95+, and CCR7+CD27+CD127+. Both Tmsc and Tcm are characterized as having weak effector T cell function, robust proliferation, robust engraftment, and long telomeres.

In certain embodiments, the CD8 cells disclosed herein show improved properties (*e.g.*, killing activity, cell proliferation, secretion of cytokines, LCK affinity, persistence, tumor infiltration ability) about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 100%, about 150%, about 200%, about 250%, about 300%, about 350%, about 400%, about 450%, or about 500% as compared to the cells that do not have a CD8 construct.

METHODS OF PRODUCING CD8 PRODUCTS WITH A YOUNG PHENOTYPE

In certain embodiments, the present disclosure relates, in part, on the production of engineered “young” T cells. In certain embodiments, the present disclosure comprises methods for producing antigen-specific cells, e.g., T cells, *ex vivo*, comprising activating,

engineering, and expanding antigen-specific cells originally obtained from a subject or isolated from such sample.

In certain embodiments, the methods for activating cells comprise the steps of activating the TCR/CD3 complex. For example, without limitation, the T cells can be incubated and/or cultured with CD3 agonists, CD28 agonists, or a combination thereof.

In certain embodiments, engineered activated antigen-specific cells, e.g., engineered activated T cells, can be expanded by culturing the engineered activated antigen-specific cells, e.g., T cells, with cytokines, chemokine, soluble peptides, or combination thereof. In certain embodiments, the engineered activated antigen-specific cells, e.g., engineered activated T cells, can be cultured with one or more cytokines. In certain embodiments, the cytokines can be IL2, IL7, IL15, or combinations thereof. For example, engineered activated antigen-specific cells, e.g., engineered activated T cells, can be cultured with IL7 and IL15. In certain embodiments, the cytokine used in connection with the engineered activated antigen-specific cell, e.g., engineered activated T cell, culture can be present at a concentration from about 1 pg/ml to about 1 g/ml, from about 1 ng/ml to about 1 g/ml, from about 1 μg/ml to about 1 g/ml, or from about 1 mg/ml to about 1g/ml, and any values in between.

PHARMACEUTICAL FORMULATIONS.

Pharmaceutical formulations of the CD8 Product are prepared by combining the CD8 Cells in a solution that can preserve the 'young' phenotype of the cells in a cryopreserved state. Table 1 provides an example of one such pharmaceutical formulation. Alternatively, pharmaceutical formulations of the CD8 Product can be prepared by combining the CD8 Cells in a solution that can preserve the 'young' phenotype of the cells without the need to freeze or cryopreserve the product (*i.e.*, the CD8 Product is maintained in an aqueous solution or as a non-frozen/cryopreserved cell pellet).

Additional pharmaceutically acceptable carriers, buffers, stabilizers, and/or preservatives can also be added to the cryopreservation solution or the aqueous storage solution (if the CD8 Product is not cryopreserved). Any cryopreservation agent and/or media can be used to cryopreserve the CD8 Product, including but not limited to CryoStor, CryoStor CS5, CELLBANKER, and custom cryopreservation media that optionally include DMSO.

GENE-EDITING METHODS

In certain embodiments, the present disclosure involves, in part, methods of engineering human cells, e.g., engineered T cells or engineered human stem cells. In certain embodiments, the present disclosure involves, in part, methods of engineering human cells, e.g., NK cells, NKT cells, macrophages, hematopoietic stem cells (HSCs), cells derived from HSCs, or dendritic/antigen-presenting cells. In certain embodiments, such engineering involves genome editing. For example, but not by way of limitation, such genome editing can be accomplished with nucleases targeting one or more endogenous loci, e.g., TCR alpha (TCRα) locus and TCR beta (TCRβ) locus. In certain embodiments, the nucleases can generate single-stranded DNA nicks or double-stranded DNA breaks in an endogenous target sequence. In certain embodiments, the nuclease can target coding or non-coding portions of the genome, e.g., exons, introns. In certain embodiments, the nucleases contemplated herein comprise homing endonuclease, meganuclease, megaTAL nuclease, transcription activator-like effector nuclease (TALEN), zinc-finger nuclease (ZFN), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas nuclease. In certain embodiments, the nucleases can themselves be engineered, e.g., via the introduction of amino acid substitutions and/or deletions, to increase the efficiency of the cutting activity.

In certain embodiments, a CRISPR/Cas nuclease system is used to engineer human cells. In certain embodiments, the CRISPR/Cas nuclease system comprises a Cas nuclease and one or more RNAs that recruit the Cas nuclease to the endogenous target sequence, *e.g.*, single guide RNA. In certain embodiments, the Cas nuclease and the RNA are introduced in the cell separately, e.g. using different vectors or compositions, or together, e.g., in a polycistronic construct or a single protein-RNA complex. In certain embodiments, the Cas nuclease is Cas9 or Cas12a. In certain embodiments, the Cas9 polypeptide is obtained from a bacterial species including, without limitation, *Streptococcus pyogenes* or *Neisseria menengitidis*. Additional examples of CRISPR/Cas systems are known in the art. See Adli, Mazhar. "The CRISPR tool kit for genome editing and beyond." Nature communications vol. 9,1 1911 (2018), herein incorporated by reference for all that it teaches.

In certain embodiments, genome editing occurs at one or more genome loci that regulate immunological responses. In certain embodiments, the loci include, without limitation, TCR alpha (TCRα) locus, TCR beta (TCRβ) locus, TCR gamma (TCRγ), and

TCR delta (TCRδ). In certain embodiments, the loci for inserting a CD8 Construct is anywhere in the genome. In certain embodiments, the loci for inserting a CD8 Construct is the TRAC locus. In certain embodiments, the loci for inserting a CD8 Construct is one of the two TRBC loci. In certain embodiments, the locus for inserting a CD8 Construct is a locus other than the TRAC locus or TRAB loci. In certain embodiments, the loci for inserting a CD8 Construct is inserted into a gene locus wherein such gene is knocked out. By way of a non-limiting example, if the desired phenotype of a CD8 Product is the expression of a NeoTCR, the expression of a CD8 Construct, and the knockout of the TET2 gene or the AAVS1 gene, the CD8 Construct can be inserted at the TET2 locus or AAVS1 locus. In certain embodiments, the insertion of the CD8 Construct is in tandem with the NeoTCR insertion. In certain embodiments, the insertion of the CD8 Construct is a separate locus than the NeoTCR insertion.

In certain embodiments, genome editing is performed by using non-viral delivery systems. For example, a nucleic acid molecule can be introduced into a cell by administering the nucleic acid in the presence of lipofection (Feigner et al., Proc. Natl. Acad. Sci. U.S.A. 84:7413, 1987; Ono et al., Neuroscience Letters 17:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Methods in Enzymology 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu et al., Journal of Biological Chemistry 263:14621, 1988; Wu et al., Journal of Biological Chemistry 264:16985, 1989), or by micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990). Other non-viral means for gene transfer include transfection in vitro using calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell. Transplantation of normal genes into the affected tissues of a subject can also be accomplished by transferring a normal nucleic acid into a cultivatable cell type ex vivo (e.g., an autologous or heterologous primary cell or progeny thereof), after which the cell (or its descendants) are injected into a targeted tissue or are injected systemically.

In certain embodiments, genome editing is performed by using viral delivery systems. In certain embodiments, the viral methods include targeted integration (including but not limited to AAV) and random integration (including but not limited to lentiviral approaches). In certain embodiments, the viral delivery would be accomplished without integration of the nuclease. In such embodiments, the viral delivery system can be Lentiflash or another similar delivery system.

HOMOLOGY RECOMBINATION TEMPLATES

In certain embodiments, the present disclosure provides genome editing of a cell by introducing and recombining a homologous recombination (HR) template nucleic acid sequence into an endogenous locus of a cell. In certain embodiments, the HR template nucleic acid sequence is linear. In certain embodiments, the HR template nucleic acid sequence is circular. In certain embodiments, the circular HR template can be a plasmid, minicircle, or nanoplasmid. In certain embodiments, the HR template nucleic acid sequence comprises a first and a second homology arms. In certain embodiments, the homology arms can be of about 300 bases to about 2,000 bases. For example, each homology arm can be 1,000 bases. In certain embodiments, the homology arms can be homologous to a first and second endogenous sequences of the cell. In certain embodiments, the endogenous locus is a TCR locus. For example, the first and second endogenous sequences are within a TCR alpha locus or a TCR beta locus. In certain embodiments, the HR template comprises a TCR gene sequences. In non-limiting embodiments, the TCR gene sequence is a patient specific TCR gene sequence. In non-limiting embodiments, the TCR gene sequence is tumor-specific. In non-limiting embodiments, the TCR gene sequence can be identified and obtained using the methods described in PCT/US2020/017887, the content of which is herein incorporated by reference. In certain embodiments, the HR template comprises a TCR alpha gene sequence and a TCR beta gene sequence.

In certain embodiments, the HR template is a polycistronic polynucleotide. In certain embodiments, the HR template comprises sequences encoding for flexible polypeptide sequences (e.g., Gly-Ser-Gly sequence). In certain embodiments, the HR template comprises sequences encoding an internal ribosome entry site (IRES). In certain embodiments, the HR template comprises a 2A peptide (e.g., P2A, T2A, E2A, and F2A). Additional information on the HR template nucleic acids and methods of modifying a cell thereof can be found in International Patent Application no. PCT/US2018/058230, the content of which is herein incorporated by reference.

METHODS OF TREATMENT

The presently disclosed subject matter provides methods for inducing and/or increasing an immune response in a subject in need thereof. The CD8 Products can be

used for treating and/or preventing a cancer in a subject. The CD8 Products can be used for prolonging the survival of a subject suffering from a cancer. The CD8 Products can also be used for treating and/or preventing a cancer in a subject. The CD8 Products can also be used for reducing tumor burden in a subject. Such methods comprise administering the CD8 Products in an amount effective or a composition (*e.g.*, a pharmaceutical composition) comprising thereof to achieve the desired effect, be it palliation of an existing condition or prevention of recurrence. For treatment, the amount administered is an amount effective in producing the desired effect. An effective amount can be provided in one or a series of administrations. An effective amount can be provided in a bolus or by continuous perfusion.

In certain embodiments, the CD8 Products can be used for treating viral or bacterial diseases. In certain embodiments, the CD8 Products can be used for treating autoimmune diseases.

In certain embodiments, an effective amount of the CD8 Products are delivered through IV administration. In certain embodiments, the CD8 Products are delivered through IV administration in a single administration. In certain embodiments, the CD8 Products are delivered through IV administration in multiple administrations. In certain embodiments, the CD8 Products are delivered through IV administration in two or more administrations. In certain embodiments, the CD8 Products are delivered through IV administration in two administrations. In certain embodiments, the CD8 Products are delivered through IV administration in three administrations.

The presently disclosed subject matter provides methods for treating and/or preventing cancer in a subject. In certain embodiments, the method comprises administering an effective amount of CD8 Products to a subject having cancer.

Non-limiting examples of cancer include blood cancers (e.g. leukemias, lymphomas, and myelomas), ovarian cancer, breast cancer, bladder cancer, brain cancer, colon cancer, intestinal cancer, liver cancer, lung cancer, pancreatic cancer, prostate cancer, skin cancer, stomach cancer, glioblastoma, throat cancer, melanoma, neuroblastoma, adenocarcinoma, glioma, soft tissue sarcoma, and various carcinomas (including prostate and small cell lung cancer). Suitable carcinomas further include any known in the field of oncology, including, but not limited to, astrocytoma, fibrosarcoma, myxosarcoma, liposarcoma, oligodendroglioma, ependymoma, medulloblastoma, primitive neural ectodermal tumor (PNET), chondrosarcoma, osteogenic sarcoma, pancreatic ductal adenocarcinoma, small and large cell lung adenocarcinomas, chordoma,

angiosarcoma, endotheliosarcoma, squamous cell carcinoma, bronchoalveolarcarcinoma, epithelial adenocarcinoma, and liver metastases thereof, lymphangiosarcoma, lymphangioendotheliosarcoma, hepatoma, cholangiocarcinoma, synovioma, mesothelioma, Ewing's tumor, rhabdomyosarcoma, colon carcinoma, basal cell carcinoma, sweat gland carcinoma, papillary carcinoma, sebaceous gland carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, testicular tumor, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, leukemia, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease, breast tumors such as ductal and lobular adenocarcinoma, squamous and adenocarcinomas of the uterine cervix, uterine and ovarian epithelial carcinomas, prostatic adenocarcinomas, transitional squamous cell carcinoma of the bladder, B and T cell lymphomas (nodular and diffuse) plasmacytoma, acute and chronic leukemias, malignant melanoma, soft tissue sarcomas and leiomyosarcomas. In certain embodiments, the neoplasia is selected from the group consisting of blood cancers (e.g. leukemias, lymphomas, and myelomas), ovarian cancer, prostate cancer, breast cancer, bladder cancer, brain cancer, colon cancer, intestinal cancer, liver cancer, lung cancer, pancreatic cancer, prostate cancer, skin cancer, stomach cancer, glioblastoma, and throat cancer. In certain embodiments, the presently disclosed young T cells and compositions comprising thereof can be used for treating and/or preventing blood cancers (e.g., leukemias, lymphomas, and myelomas) or ovarian cancer, which are not amenable to conventional therapeutic interventions.

In certain embodiments, the neoplasia is a solid cancer or a solid tumor. In certain embodiments, the solid tumor or solid cancer is selected from the group consisting of glioblastoma, prostate adenocarcinoma, kidney papillary cell carcinoma, sarcoma, ovarian cancer, pancreatic adenocarcinoma, rectum adenocarcinoma, colon adenocarcinoma, esophageal carcinoma, uterine corpus endometrioid carcinoma, breast cancer, skin cutaneous melanoma, lung adenocarcinoma, stomach adenocarcinoma, cervical and endocervical cancer, kidney clear cell carcinoma, testicular germ cell tumors, and aggressive B-cell lymphomas.

The subjects can have an advanced form of disease, in which case the treatment objective can include mitigation or reversal of disease progression, and/or amelioration of side effects. The subjects can have a history of the condition, for which they have already

been treated, in which case the therapeutic objective will typically include a decrease or delay in the risk of recurrence.

Suitable human subjects for therapy typically comprise two treatment groups that can be distinguished by clinical criteria. Subjects with "advanced disease" or "high tumor burden" are those who bear a clinically measurable tumor. A clinically measurable tumor is one that can be detected on the basis of tumor mass (e.g., by palpation, CAT scan, sonogram, mammogram or X-ray; positive biochemical or histopathologic markers on their own are insufficient to identify this population). A pharmaceutical composition is administered to these subjects to elicit an anti-tumor response, with the objective of palliating their condition. Ideally, reduction in tumor mass occurs as a result, but any clinical improvement constitutes a benefit. Clinical improvement includes decreased risk or rate of progression or reduction in pathological consequences of the tumor.

ARTICLES OF MANUFACTURE

The CD8 Products can be used in combination with articles of manufacture. Such articles of manufacture can be useful for the prevention or treatment of proliferative disorders (e.g., cancer). Examples of articles of manufacture include but are not limited to containers (e.g., infusion bags, bottles, storage containers, flasks, vials, syringes, tubes, and IV solution bags) and a label or package insert on or associated with the container. The containers may be made of any material that is acceptable for the storage and preservation of the CD8 Cells within the CD8 Products. In certain embodiments, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle. For example, the container may be a CryoMACS freezing bag. The label or package insert indicates that the CD8 Products are used for treating the condition of choice and the patient of origin. The patient is identified on the container of the CD8 Product because the CD8 Products is made from autologous cells and engineered as a patient-specific and individualized treatment.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein; and 2) a second container with the same CD8 Product as the first container contained therein. Optionally, additional containers with the same CD8 Product as the first and second containers may be prepared and made. Optionally, additional

containers containing a composition comprising a different cytotoxic or otherwise therapeutic agent may also be combined with the containers described above.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein; and 2) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with two CD8 Products contained therein; and 2) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein; 2) a second container with a second CD8 Product contained therein; and 3) optionally a third container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. In certain embodiments, the first and second CD8 Products are different CD8 Products. In certain embodiments, the first and second CD8 Products are the same CD8 Products.

The article of manufacture may comprise: 1) a first container with three CD8 Products contained therein; and 2) optionally a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein; 2) a second container with a second CD8 Product contained therein; 3) a third container with a third CD8 Product contained therein; and 4) optionally a fourth container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. In certain embodiments, the first, second, and third CD8 Products are different CD8 Products. In certain embodiments, the first, second, and third CD8 Products are the same CD8 Products. In certain embodiments, two of the first, second, and third CD8 Products are the same CD8 Products.

The article of manufacture may comprise: 1) a first container with four CD8 Products contained therein; and 2) optionally a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein; 2) a second container with a second CD8 Product contained therein; 3) a third container with a third CD8 Product contained therein; 4) a fourth container with a

fourth CD8 Product contained therein; and 5) optionally a fifth container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. In certain embodiments, the first, second, third, and fourth CD8 Products are different CD8 Products. In certain embodiments, the first, second, third, and fourth CD8 Products are the same NeoTCR Products. In certain embodiments, two of the first, second, third, and fourth CD8 Products are the same NeoTCR Products. In certain embodiments, three of the first, second, third, and fourth CD8 Products are the same CD8 Products.

The article of manufacture may comprise: 1) a first container with five or more CD8 Products contained therein; and 2) optionally a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein; 2) a second container with a second CD8 Product contained therein; 3) a third container with a third CD8 Product contained therein; 4) a fourth container with a fourth CD8 Product contained therein; 5) a fifth container with a fifth CD8 Product contained therein; 6) optionally a sixth or more additional containers with a sixth or more CD8 Product contained therein; and 7) optionally an additional container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. In certain embodiments, all of the containers of CD8 Products are different CD8 Products. In certain embodiments, all of the containers of CD8 Products are the same CD8 Products. In certain embodiments, there can be any combination of same or different CD8 Products in the five or more containers based on the availability of detectable CD8s in a patient's tumor sample(s), the need and/or desire to have multiple CD8 Products for the patient, and the availability of any one CD8 Product that may require or benefit from one or more container.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein; 2) a second container with a second CD8 Product contained therein; and 3) a third container with a third CD8 Product contained therein.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein; 2) a second container with a second CD8 Product contained therein; 3) a third container with a third CD8 Product contained therein; and 4) optionally a fourth container with a fourth CD8 Product contained therein.

The article of manufacture may comprise: 1) a first container with a CD8 Product contained therein; 2) a second container with a second CD8 Product contained therein; 3) a third container with a third CD8 Product contained therein; 4) a fourth container with a fourth CD8 Product contained therein; and 5) optionally a fifth container with a fourth CD8 Product contained therein.

The article of manufacture may comprise a container with one CD8 Product contained therein. The article of manufacture may comprise a container with two CD8 Products contained therein. The article of manufacture may comprise a container with three CD8 Products contained therein. The article of manufacture may comprise a container with four CD8 Products contained therein. The article of manufacture may comprise a container with five CD8 Products contained therein.

The article of manufacture may comprise 1) a first container with one CD8 Product contained therein, and 2) a second container with two CD8 Products contained therein. The article of manufacture may comprise 1) a first container with two CD8 Products contained therein, and 2) a second container with one CD8 Product contained therein. In the examples above, a third and/or fourth container comprising one or more additional CD8 Products may be included in the article of manufacture. Additionally, a fifth container comprising one or more additional CD8 Products may be included in the article of manufacture.

Furthermore, any container of CD8 Product described herein can be split into two, three, or four separate containers for multiple time points of administration and/or based on the appropriate dose for the patient.

In certain embodiments, the CD8 Products are provided in a kit. The kit can, by means of non-limiting examples, contain package insert(s), labels, instructions for using the CD8 Product(s), syringes, disposal instructions, administration instructions, tubing, needles, and anything else a clinician would need in order to properly administer the CD8 Product(s).

THERAPEUTIC COMPOSITIONS AND METHODS OF MANUFACTURING

As described herein, plasmid DNA-mediated precision genome engineering process for Good Manufacturing Practice (GMP) manufacturing of CD8 Products was developed. Targeted integration of the patient-specific neoTCR was accomplished by electroporating CRISPR endonuclease ribonucleoproteins (RNPs) together with the

personalized neoTCR gene cassette, encoded by the plasmid DNA. In addition to the neoTCR, the CD8 Constructs were inserted by incorporating them into the neoTCR vector and then electroporating with CRISPR endonuclease ribonucleoproteins (RNPs) as described above.

The CD8 Products can be formulated into a drug product using the clinical manufacturing process. Under this process, the CD8 Products are cryopreserved in CryoMACS Freezing Bags. One or more bags may be shipped to the site for each patient depending on patient needs. The product is composed of apheresis-derived, patient-autologous, CD8 and CD4 T cells that have been precision genome engineered to express one or more autologous neoTCRs targeting a neoepitope complexed to one of the endogenous HLA receptors presented exclusively on the surface of that patient's tumor cells.

The final product will contain 5% dimethyl sulfoxide (DMSO), human serum albumin, and Plasma-Lyte. The final cell product will contain the list of components provided in **Table 2**. **Composition of the CD8 Product**

Component	Specification/Grade
Total nucleated NeoTCR cells	cGMP manufactured
Plasma-Lyte A	USP
Human Serum Albumin in 0.02 - 0.08 M sodium caprylate and sodium tryptophanate	USP
CryoStor CS10	cGMP manufactured with USP grade materials

COMPOSITIONS AND VECTORS

The presently disclosed subject matter provides compositions comprising cells (e.g., immunoresponsive cells) disclosed herein.

In certain embodiments, the presently disclosed subject matter provides nucleic acid compositions comprising a polynucleotide encoding the NeoTCR disclosed herein. In certain embodiments, the nucleic acid compositions disclosed herein comprise a polynucleotide encoding a CD8 Construct disclosed herein. Also provided are cells comprising such nucleic acid compositions.

In certain embodiments, the nucleic acid composition further comprises a promoter that is operably linked to the NeoTCR disclosed herein. In certain embodiments, the

nucleic acid composition further comprises a promoter that is operably linked to the CD8 Construct disclosed herein.

In certain embodiments, the promoter is endogenous or exogenous. In certain embodiments, the exogenous promoter is selected from the group consisting of an elongation factor (EF)-1 promoter, a CMV promoter, a SV40 promoter, a PGK promoter, a long terminal repeat (LTR) promoter and a metallothionein promoter. In certain embodiments, the promoter is an inducible promoter. In certain embodiments, the inducible promoter is selected from the group consisting of a NFAT transcriptional response element (TRE) promoter, a CD69 promoter, a CD25 promoter, an IL-2 promoter, an IL-12 promoter, a p40 promoter, and a Bcl-xL promoter.

The compositions and nucleic acid compositions can be administered to subjects or and/delivered into cells by art-known methods or as described herein. Genetic modification of a cell (e.g., a T cell) can be accomplished by transducing a substantially homogeneous cell composition with a recombinant DNA construct. In certain embodiments, a retroviral vector (either a gamma-retroviral vector or a lentiviral vector) is employed for the introduction of the DNA construct into the cell. Non-viral vectors may be used as well.

Possible methods of transduction also include direct co-culture of the cells with producer cells, e.g., by the method of Bregni, *et al.* (1992) *Blood* 80:1418-1422, or culturing with viral supernatant alone or concentrated vector stocks with or without appropriate growth factors and polycations, e.g., by the method of Xu, *et al.* (1994) *Exp. Hemat.* 22:223-230; and Hughes, *et al.* (1992) *J. Clin. Invest.* 89:1817.

Other transducing viral vectors can be used to modify a cell. In certain embodiments, the chosen vector exhibits high efficiency of infection and stable integration and expression (see, e.g., Cayouette et al., Human Gene Therapy 8:423-430, 1997; Kido et al., Current Eye Research 15:833-844, 1996; Bloomer et al., Journal of Virology 71:6641-6649, 1997; Naldini et al., Science 272:263-267, 1996; and Miyoshi et al., Proc. Natl. Acad. Sci. U.S.A. 94:10319, 1997). Other viral vectors that can be used include, for example, adenoviral, lentiviral, and adena-associated viral vectors, vaccinia virus, a bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus (also see, for example, the vectors of Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis et al., BioTechniques 6:608-614, 1988; Tolstoshev et al., Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science

226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller et al., Biotechnology 7:980-990, 1989; LeGal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S- 83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson et al., U.S. Pat. No. 5,399,346).

Non-viral approaches can also be employed for genetic modification of a cell. For example, a nucleic acid molecule can be introduced into a cell by administering the nucleic acid in the presence of lipofection (Feigner et al., Proc. Natl. Acad. Sci. U.S.A. 84:7413, 1987; Ono et al., Neuroscience Letters 17:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Methods in Enzymology 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu et al., Journal of Biological Chemistry 263:14621, 1988; Wu et al., Journal of Biological Chemistry 264:16985, 1989), or by micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990). Other non-viral means for gene transfer include transfection *in vitro* using calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell. Transplantation of normal genes into the affected tissues of a subject can also be accomplished by transferring a normal nucleic acid into a cultivatable cell type *ex vivo* (e.g., an autologous or heterologous primary cell or progeny thereof), after which the cell (or its descendants) are injected into a targeted tissue or are injected systemically.

Polynucleotide therapy methods can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element or intron (e.g. the elongation factor 1a enhancer/promoter/intron structure). For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct the expression of a nucleic acid. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers. Alternatively, if a genomic clone is used as a therapeutic construct, regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

The resulting cells can be grown under conditions similar to those for unmodified cells, whereby the modified cells can be expanded and used for a variety of purposes.

KITS

The presently disclosed subject matter provides kits for inducing and/or enhancing an immune response and/or treating and/or preventing a cancer or a pathogen infection in a subject. In certain embodiments, the kit comprises an effective amount of presently disclosed cells or a pharmaceutical composition comprising thereof. In certain embodiments, the kit comprises a sterile container; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments. In certain non-limiting embodiments, the kit includes an isolated nucleic acid molecule encoding a presently disclosed HR template.

If desired, the cells and/or nucleic acid molecules are provided together with instructions for administering the cells or nucleic acid molecules to a subject having or at risk of developing a cancer or pathogen or immune disorder. The instructions generally include information about the use of the composition for the treatment and/or prevention of a cancer or a pathogen infection. In certain embodiments, the instructions include at least one of the following: description of the therapeutic agent; dosage schedule and administration for treatment or prevention of a neoplasia, pathogen infection, or immune disorder or symptoms thereof; precautions; warnings; indications; counter-indications; over-dosage information; adverse reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container. The resulting cells can be grown under conditions similar to those for unmodified cells, whereby the modified cells can be expanded and used for a variety of purposes.

EXEMPLARY EMBODIMENTS

A. In certain non-limiting embodiments, the presently disclosed subject matter provides for a cell, comprising an exogenous T cell receptor (TCR), and an exogenous CD8.

A1. The foregoing of A, wherein the exogenous CD8 comprises at least one monomer.

A2. The foregoing cell of A1, wherein the at least one monomer of the exogenous CD8 comprises an extracellular domain, a transmembrane domain, an intracellular domain, fragments thereof, or combinations thereof.

A3. The foregoing cell of A2, wherein the extracellular domain comprises a CD8α extracellular domain or a CD8β extracellular domain.

A4. The foregoing cell of A2 or A3, wherein the transmembrane a CD8α transmembrane domain or a CD8β transmembrane domain.

A5. The foregoing cell of A2-A4, wherein the intracellular domain comprises a CD8α intracellular domain or a CD8β intracellular domain.

A6. The foregoing cell of A2-A4, wherein the intracellular domain comprises a CD4 intracellular domain.

A7. The foregoing cell of A1-A5, wherein the at least one monomer comprises a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain.

A8. The foregoing cell of A1-A5, wherein the at least one monomer comprises a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain.

A9. The foregoing cell of A1-A5, wherein the at least one monomer comprises a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain.

A10. The foregoing cell of A1-A6, wherein the at least one monomer comprises a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

A11. The foregoing cell of A1-A10, wherein the at least one monomer comprises a signal peptide.

A12. The foregoing cell of A11, wherein the signal peptide is a CD8 signal peptide.

A13. The foregoing cell of A2-A12, wherein the extracellular domain comprises the amino acid sequence set forth in SEQ ID NO: 140, or SEQ ID NO: 145.

A14. The foregoing cell of A2-A13, wherein the transmembrane domain comprises the amino acid sequence set forth in SEQ ID NO: 141, or SEQ ID NO: 146.

A15. The foregoing cell of A2-A14, wherein the intracellular domain comprises the amino acid sequence set forth in SEQ ID NO: 142, SEQ ID NO: 147, or SEQ ID NO: 148.

A16. The foregoing cell of A11-A15, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO: 139, or SEQ ID NO: 144.

A17. The foregoing cell of A-A16, wherein the exogenous CD8 comprises a 2A sequence.

A18. The foregoing cell of A-A17, wherein the exogenous CD8 comprises a linker.

A19. The foregoing cell of A18, wherein the linker comprises the amino acid sequence set forth in SEQ ID NO: 137.

A20. The foregoing cell of A-A19, wherein the exogenous CD8 comprises a protease cleavage site.

A21. The foregoing cell of A-A20, wherein the protease cleavage site is a Furin cleavage site.

A22. The foregoing cell of A-A21, wherein the exogenous TCR is a patient derived TCR.

A23. The foregoing cell of A-A22, wherein the exogenous TCR comprises a signal sequence, a first and second 2A sequence, and a TCR polypeptide sequence.

A24. The foregoing cell of A-A23, wherein the exogenous TCR recognizes a cancer antigen.

A25. The foregoing cell of A24, wherein the cancer antigen is a neoantigen.

A26. The foregoing cell of A24, wherein the cancer antigen is a patient specific antigen.

A27. The foregoing cell of A-A26, wherein the cell is a primary cell.

A28. The foregoing cell of A-A26, wherein the cell is a patient-derived cell.

A29. The foregoing cell of A-A26, wherein the cell is a lymphocyte.

A30. The foregoing cell of A-A26, wherein the cell is a T cell.

A31. The foregoing cell of A-A26, wherein the cell if a young T cell.

A32. The foregoing cell of A31, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.

A33. The foregoing cell of A31, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.

A34. The foregoing cell of A31, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

A35. The foregoing cell of A-A34, further comprising a gene modification to enhance cell persistence and/or enhances memory cell differentiation

A36. The foregoing cell of A-A35, wherein killing activity of the cell is increased between about 10% to about 500% as compared to killing activity of a cell that does not have the exogenous CD8.

A37. The foregoing cell of A-A36, wherein proliferation of the cell upon binding of the TCR to the antigen is increased between about 10% to about 500% as compared to proliferation of a cell that does not have the exogenous CD8.

A38. The foregoing cell of A-A37, wherein secretion of pro-inflammatory cytokine upon binding of the TCR to the antigen by the cell is increased between about 10% to about 500% as compared to secretion by a cell that does not have the exogenous CD8.

A39. The foregoing cell of A-A38, wherein LCK affinity of the cell is increased between about 10% to about 500% as compared to LCK affinity of a cell that does not have the exogenous CD8.

A40. The foregoing cell of A-A39, wherein persistence of the cell is increased between about 10% to about 500% as compared to persistence of a cell that does not have the exogenous CD8.

A41. The foregoing cell of A-A40, wherein tumor infiltration ability of the cell is increased between about 10% to about 500% as compared to tumor infiltration ability of a cell that does not have the exogenous CD8.

A42. The foregoing cell of A-A41, wherein the exogenous TCR is a CD8-dependent TCR.

A43. The foregoing cell of A-A41, wherein the exogenous TCR is a CD8-independent TCR.

A44. The foregoing cell of A-A43, wherein the exogenous CD8 is encoded by a CD8 Construct 1, a CD8 Construct 2, a CD8 Construct 3, or a CD8 Construct 4.

A45. The foregoing cell of A-A43, wherein the exogenous CD8 comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane

domain, and aCD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

A46. The foregoing cell of A-A43, wherein the exogenous CD8 comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

B. In certain non-limiting embodiments, the presently disclosed subject matter provides for a method of modifying a cell, the method comprising introducing into the cell a homologous recombination (HR) template nucleic acid sequence, wherein the HR template comprises first and second homology arms homologous to first and second target nucleic acid sequences, a TCR gene sequence positioned between the first and second homology arms, and a CD8 gene sequence positioned between the first and the second homology arms, and recombining the HR template nucleic acid into an endogenous locus of the cell.

B1. The foregoing method of B, wherein the HR template comprises a first 2A-coding sequence positioned upstream of the CD8 gene sequence, a second 2A-coding sequence positioned downstream of the CD8 gene sequence and upstream of the TCR gene sequence, and a third 2A-coding sequence positioned downstream of the TCR gene sequence; wherein the first, second, and third 2A-coding sequences code for the same amino acid sequence and are codon-diverged relative to each other.

B2. The foregoing method of B or B1, wherein the HR template comprises a sequence coding for the amino acid sequence Gly Ser Gly positioned immediately upstream of the first, second, and/or third 2A-coding sequences.

B3. The foregoing method of B1 or B2, wherein the HR template further comprises a sequence coding for a Furin cleavage site positioned upstream of the first, second, and/or third 2A-coding sequences.

B4. The foregoing method of B-B3, wherein the HR template further comprises a sequence encoding a signal sequence positioned immediately upstream of the TCR gene sequence and/or the CD8 gene sequence.

B5. The foregoing method of B-B4, wherein the HR template comprises a second TCR sequence positioned between the third 2A-coding sequence and the second homology arm.

B6. The foregoing method of B5, wherein the HR template comprises a sequence encoding a first signal sequence positioned immediately upstream the first TCR gene sequence; and a sequence encoding a second signal sequence positioned immediately upstream of the second TCR gene sequence.

B7. The foregoing method of B-B6, wherein the HR template comprises a second CD8 gene sequence positioned between the first CD8 gene sequence and the second 2A-coding sequence.

B8. The foregoing method of B7, wherein a 2A coding sequence is positioned between the first and second CD8 gene sequence.

B9. The foregoing method of B7 or B8, wherein a sequence coding for the amino acid sequence Gly Ser Gly is positioned between the first and second CD8 gene sequences.

B10. The foregoing method of B7-B9, wherein a sequence coding for a Furin cleavage site is positioned between the first and second CD8 gene sequences.

B11. The foregoing method of B-B10, wherein the CD8 gene sequence comprises a sequence encoding an extracellular domain, a sequence encoding an intracellular domain, a sequence encoding an intracellular domain, fragments thereof, or combinations thereof.

B12. The foregoing method of B11, wherein the sequence encoding an extracellular domain comprises a sequence encoding a CD8α extracellular domain or a CD8β extracellular domain.

B13. The foregoing method of B11 or B12, wherein the sequence encoding a transmembrane domain comprises a sequence encoding a CD8α transmembrane domain or a CD8β transmembrane domain.

B14. The foregoing method of B11-B13, wherein the sequence encoding an intracellular domain comprises a sequence encoding a CD8α intracellular domain or a CD8β intracellular domain.

B15. The foregoing method of B11-B14, wherein the sequence encoding an intracellular domain comprises a sequence encoding a CD4 intracellular domain.

B16. The foregoing method of B11-B15, wherein the CD8 gene sequence comprises a sequence encoding a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain.

B17. The foregoing method of B11-B15, wherein the CD8 gene sequence comprises a sequence encoding a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain.

B18. The foregoing method of B11-B15, wherein the CD8 gene sequence comprises a sequence encoding a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain.

B19. The foregoing method of B11-B15, wherein the CD8 gene sequence comprises a sequence encoding a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

B20. The foregoing method of B7-B19, wherein the HR template comprises a sequence encoding a first signal sequence positioned immediately upstream the first CD8 gene sequence; and a sequence encoding a second signal sequence positioned immediately upstream of the second CD8 gene sequence.

B21. The foregoing method of B4-B20, wherein the signal sequence is a CD8 signal sequence, a human growth hormone signal sequence, fragments thereof, or combinations thereof.

B22. The foregoing method of B-B21, wherein the first and second homology arms of the HR template are each from about 300 bases to about 2,000 bases in length.

B23. The foregoing method of B-B22, wherein the first and second homology arms of the HR template are each from about 600 bases to about 2,000 bases in length.

B24. The foregoing method of B-B23, wherein the exogenous TCR is a patient derived TCR.

B25. The foregoing method of B-B24, wherein the exogenous TCR comprises a signal sequence, a first and second 2A sequence, and a TCR polypeptide sequence.

B26. The foregoing method of B-B25, wherein the exogenous TCR recognizes a cancer antigen.

B27. The foregoing method of B26, wherein the cancer antigen is a neoantigen.

B28. The foregoing method of B26, wherein the cancer antigen is a patient specific antigen.

B29. The foregoing method of B-B28, wherein the HR template is non-viral.

B30. The foregoing method of B-B29, wherein the HR template is a circular DNA.

B31. The foregoing method of B-B29, wherein the HR template is a linear DNA.

B32. The foregoing method of B-B31, wherein the introducing occurs via electroporation.

B33. The foregoing method of B-B32, wherein the recombining comprises cleavage of the endogenous locus by a nuclease; and recombination of the HR template nucleic acid sequence into the endogenous locus by homology directed repair.

B34. The foregoing method of B33, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.

B35. The foregoing method of B34, further comprising a gRNA.

B36. The foregoing method of B-B35, wherein the process further comprises culturing the cell.

B37. The foregoing method of B36, wherein the culturing is conducted in the presence of at least one cytokine.

B38. The foregoing method of B36 or B37, wherein the culturing is conducted in the presence of IL2, IL7, IL15, or any combination thereof.

B39. The foregoing method of B36 or B37, wherein the culturing is conducted in the presence of IL7 and IL15.

B40. The foregoing method of B-B39, further comprising a gene modification to enhance cell persistence and/or enhances memory cell differentiation.

B41. The foregoing method of B-B40, wherein the cell is a primary cell.

B42. The foregoing method of B-B40, wherein the cell is a patient-derived cell.

B43. The foregoing method of B-B40, wherein the cell is a lymphocyte.

B44. The foregoing method of B-B40, wherein the cell is a T cell.

B45. The foregoing method of B-B40, wherein the cell is a young T cell.

B46. The foregoing method of B45, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.

B47. The foregoing method of B45, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.

B48. The foregoing method of B45, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

B49. The foregoing method of B-B48, wherein killing activity of the cell is increased between about 10% to about 500% as compared to killing activity of a cell that does not have the CD8 gene sequence.

B50. The foregoing method of B-B49, wherein proliferation of the cell upon binding of the TCR to the antigen is increased between about 10% to about 500% as compared to proliferation of a cell that does not have the CD8 gene sequence.

B51. The foregoing method of B-B50, wherein secretion of pro-inflammatory cytokine upon binding of the TCR to the antigen by the cell is increased between about 10% to about 500% as compared to secretion by a cell that does not have the CD8 gene sequence.

B52. The foregoing method of B-B51, wherein LCK affinity of the cell is increased between about 10% to about 500% as compared to LCK affinity of a cell that does not have the CD8 gene sequence.

B53. The foregoing method of B-B52, wherein persistence of the cell is increased between about 10% to about 500% as compared to persistence of a cell that does not have the CD8 gene sequence.

B54. The foregoing method of B-B53, wherein tumor infiltration ability of the cell is increased between about 10% to about 500% as compared to tumor infiltration ability of a cell that does not have the CD8 gene sequence.

B55. The foregoing method of B-B54, wherein the TCR gene encodes a CD8-dependent TCR.

B56. The foregoing method of B-B54, wherein the TCR gene encodes a CD8-independent TCR.

B57. The foregoing method of B-B56, wherein the CD8 gene sequence is encoded by a CD8 Construct 1, a CD8 Construct 2, a CD8 Construct 3, or a CD8 Construct 4.

B58. The foregoing method of B-B56, wherein the CD8 gene sequence comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or a CD8α extracellular domain, CD8α transmembrane domain, CD4 intracellular domain.

B59. The foregoing method of B-B56, wherein the CD8 gene sequence comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

C. In certain non-limiting embodiments, the presently disclosed subject matter provides for a cell modified by the method of B-B57.

D. In certain non-limiting embodiments, the presently disclosed subject matter provides for a composition comprising an effective amount of a cell of A-A46 or a cell of C.

D1. The foregoing composition of D, wherein the composition is a pharmaceutical composition that further comprises a pharmaceutically acceptable excipient.

D2. The foregoing composition of D or D1, wherein the composition is administered to a patient in need thereof for the treatment of cancer.

D3. The foregoing composition of D-D2, wherein the composition comprises a cryopreservation agent.

D4. The foregoing composition of D-D3, wherein the composition comprises serum albumin.

D5. The foregoing composition of D-D4, wherein the composition comprises Plasma-Lyte A, HSA, and CryoStor CS10.

E. In certain non-limiting embodiments, the presently disclosed subject matter provides for a method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell of A-A46, a cell of claim C, or a composition of D-D5.

E1. The foregoing method of E, wherein prior to administering the therapeutically effective amount of cells, a non-myeloablative lymphodepletion regimen is administered to the subject.

E2. The foregoing method of E or E1, wherein the cancer is a solid tumor.

E3. The foregoing method of E or E1, wherein the cancer is liquid tumor.

E4. The foregoing method of E2, wherein the solid tumor is selected from the group consisting of melanoma, thoracic cancer, lung cancer, ovarian cancer, breast cancer, pancreatic cancer, head and neck cancer, prostate cancer, gynecological cancer, central nervous system cancer, cutaneous cancer, HPV+ cancer, esophageal cancer, thyroid cancer, gastric cancer, hepatocellular cancer, cholangiocarcinomas, renal cell cancers, testicular cancer, sarcomas, and colorectal cancer.

E5. The foregoing method of E3, wherein the liquid tumor is selected from the group consisting of follicular lymphoma, leukemia, and multiple myeloma.

F. In certain non-limiting embodiments, the presently disclosed subject matter provides for a kit comprising a cell of A-A46, reagents for performing the method of B-B57, a cell of C, or a composition of D-D5.

F1. The foregoing kit of F, wherein the kit further comprises written instructions for treating a cancer.

G. In certain non-limiting embodiments, the presently disclosed subject matter provides for a cell, comprising: an exogenous T cell receptor (TCR); and an exogenous CD8, comprising: a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

H. In certain non-limiting embodiments, the presently disclosed subject matter provides for a cell, comprising: an exogenous T cell receptor (TCR); and an exogenous CD8, comprising: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane

domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

I. In certain non-limiting embodiments, the presently disclosed subject matter provides for a method of modifying a cell, the method comprising: introducing into the cell a homologous recombination (HR) template nucleic acid sequence, wherein the HR template comprises: first and second homology arms homologous to first and second target nucleic acid sequences; a TCR gene sequence positioned between the first and second homology arms; a CD8 gene sequence positioned between the first and the second homology arms; and recombining the HR template nucleic acid into an endogenous locus of the cell, wherein the CD8 gene sequence comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

J. In certain non-limiting embodiments, the presently disclosed subject matter provides for a method of modifying a cell, the method comprising: introducing into the cell a homologous recombination (HR) template nucleic acid sequence, wherein the HR template comprises: first and second homology arms homologous to first and second target nucleic acid sequences; a TCR gene sequence positioned between the first and second homology arms; a CD8 gene sequence positioned between the first and the second homology arms; and recombining the HR template nucleic acid into an endogenous locus of the cell, wherein the CD8 gene sequence comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular

domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

K. In certain non-limiting embodiments, the presently disclosed subject matter provides for a composition comprising a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

L. In certain non-limiting embodiments, the presently disclosed subject matter provides for a composition comprising a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

M. In certain non-limiting embodiments, the presently disclosed subject matter provides for a method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the

exogenous CD8 comprises: a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain; a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

N. A method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises: a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147); a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

EXAMPLES

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

Example 1. Generation of NeoTCR Products

Neoepitope-specific TCRs identified by the imPACT Isolation Technology described in PCT/US2020/17887 (which is herein incorporated by reference in its entirety) were used to generate homologous recombination (HR) DNA templates. These HR templates were transfected into primary human T cells in tandem with site-specific nucleases (*see* **Figures 1A-1C**). The single-step non-viral precision genome engineering

resulted in the seamless replacement of the endogenous TCR with the patient's neoepitope-specific TCR, expressed by the endogenous promoter. The TCR expressed on the surface is entirely native in sequence.

The precision of neoTCR-T cell genome engineering was evaluated by Targeted Locus Amplification (TLA) for off-target integration hot spots or translocations, and by next generation sequencing based off-target cleavage assays and found to lack evidence of unintended outcomes.

As shown in **Figures 1A-1C**, constructs containing genes of interest were inserted into endogenous loci. This was accomplished with the use of homologous repair templates containing the coding sequence of the gene of interest flanked by left and right HR arms. In addition to the HR arms, the gene of interest was sandwiched between 2A peptides, a protease cleavage site that is upstream of the 2A peptide to remove the 2A peptide from the upstream translated gene of interest, and signal sequences (**Figure 1B**). Once integrated into the genome, the gene of interested expression gene cassette was transcribed as single messenger RNA. During the translation of this gene of interest in messenger RNA, the flanking regions were unlinked from the gene of interest by the self-cleaving 2A peptide and the protease cleavage site was cleaved for the removal of the 2A peptide upstream from the translated gene of interest (**Figure 1C**). In addition to the 2A peptide and protease cleavage site, a gly-ser-gly (GSG) linker was inserted before each 2A peptide to further enhance the separation of the gene of interest from the other elements in the expression cassette.

It was determined that P2A peptides were superior to other 2A peptides for Cell Products because of its efficient cleavage. Accordingly, two (2) P2A peptides and codon divergence were used to express the gene of interest without introducing any exogenous epitopes from remaining amino acids on either end of the gene of interest from the P2A peptide. The benefit of the gene edited cell having no exogenous epitopes (i.e., no flanking P2A peptide amino acids on either side of the gene of interest) is that immunogenicity is drastically decreased and there is less likelihood of a patient infused with a Cell Product containing the gene edited cell to have an immune reaction against the gene edited cell.

As described in PCT/US/2018/058230, NeoTCRs were integrated into the TCRα locus of T cells. Specifically, a homologous repair template containing a NeoTCR coding sequence flanked by left and right HR Arms was used. In addition, the endogenous TCRβ locus was disrupted leading to the expression of only TCR sequences encoded by the

NeoTCR construct. The general strategy was applied using circular HR templates as well as with linear templates.

The target TCRα locus (Cα) is shown along with the plasmid HR template, and the resulting edited sequence and downstream mRNA/protein products in **Figures 1B and 1C**. The target TCRα locus (endogenous TRAC) and its CRISPR Cas9 target site (horizontal stripe, cleavage site designated by arrow) are shown (**Figures 1A-1C**). The circular plasmid HR template with the polynucleotide encoding the NeoTCR is located between left and right homology arms ("LHA" and "RHA" respectively). The region of the TRAC introduced by the HR template that was codon optimized is shown (vertical stripe). The TCRβ constant domain was derived from TRBC2, which is indicated as being functionally equivalent to TRBC1. Other elements in the NeoTCR cassette include: 2A = 2A ribosome skipping element (by way of non-limiting example, the 2A peptides used in the cassette are both P2A sequences that are used in combination with codon divergence to eliminate any otherwise occurring non-endogenous epitopes in the translated product); P = protease cleavage site upstream of 2A that removes the 2A tag from the upstream TCRβ protein (by way of non-limiting example the protease cleavage site can be a furin protease cleavage site); SS = signal sequences (by way of non-limited example the protease cleavage site can be a human growth hormone signal sequence). The HR template of the NeoTCR expression gene cassette includes two flanking homology arms to direct insertion into the TCRα genomic locus targeted by the CRISPR Cas9 nuclease RNP with the TCRα guide RNA. These homology arms (LHA and RHA) flank the neoE-specific TCR sequences of the NeoTCR expression gene cassette. While the protease cleavage site used in this example was a furin protease cleavage site, any appropriate protease cleavage site known to one of skill in the art could be used. Similarly, while HGH was the signal sequence chosen for this example, any signal sequence known to one of skill in the art could be selected based on the desired trafficking and used.

Once integrated into the genome (**Figure 1C**), the NeoTCR expression gene cassette is transcribed as a single messenger RNA from the endogenous TCRα promoter, which still includes a portion of the endogenous TCRα polypeptide from that individual T cell (**Figure 1C**). During ribosomal polypeptide translation of this single NeoTCR messenger RNA, the NeoTCR sequences are unlinked from the endogenous, CRISPR-disrupted TCRα polypeptide by self-cleavage at a P2A peptide (**Figure 1C**). The encoded NeoTCRα and NeoTCRβ polypeptides are also unlinked from each other through cleavage by the endogenous cellular human furin protease and a second self-cleaving P2A sequence

motifs included in the NeoTCR expression gene cassette (**Figure 1C**). The NeoTCRα and NeoTCRβ polypeptides are separately targeted by signal leader sequences (derived from the human growth hormone, HGH) to the endoplasmic reticulum for multimer assembly and trafficking of the NeoTCR protein complexes to the T cell surface. The inclusion of the furin protease cleavage site facilitates the removal of the 2A sequence from the upstream TCRβ chain to reduce potential interference with TCRβ function. Inclusion of a gly-ser-gly linker before each 2A (not shown) further enhances the separation of the three polypeptides.

Additionally, three repeated protein sequences are codon diverged within the HR template to promote genomic stability. The two P2A are codon diverged relative to each other, as well as the two HGH signal sequences relative to each other, within the TCR gene cassette to promote stability of the introduced NeoTCR cassette sequences within the genome of the *ex vivo* engineered T cells. Similarly, the re-introduced 5' end of TRAC exon 1 (vertical stripe) reduces the likelihood of the entire cassette being lost over time through the removal of intervening sequence of two direct repeats.

In addition to NeoTCR Products, this method can be used for any CD8 Product.

In-Out PCR was used to confirm the precise target integration of the NeoE TCR cassette. Agarose gels show the results of a PCR using primers specific to the integration cassette and site generate products of the expected size only for cells treated with both nuclease and DNA template (KOKI and KOKIKO), demonstrating site-specific and precise integration.

Furthermore, Targeted Locus Amplification (TLA) was used to confirm the specificity of targeted integration. Crosslinking, ligation, and use of primers specific to the NeoTCR insert were used to obtain sequences around the site(s) of integration. The reads mapped to the genome are binned in 10 kb intervals. Significant read depths were obtained only around the intended site the integration site on chromosome 14, showing no evidence of common off-target insertion sites.

Antibody staining for endogenous TCR and peptide-HLA staining for neoTCR revealed that the engineering results in high frequency knock-in of the NeoTCR, with some TCR- cells and few WT T cells remaining. Knock-in is evidenced by neoTCR expression in the absence of an exogenous promoter. Engineering was carried out multiple times using the same neoTCR with similar results. Therefore, efficient and consistent expression of the NeoTCR and knockout of the endogenous TCR in engineered T cells was achieved.

Example 2. Generation of CD8 Product 1

T cell Isolation and Editing. CD4 and CD8 T cells were isolated from healthy donor PBMCs using the Miltenyi Prodigy or Miltenyi MACS separation columns according to the manufacturers' instructions. Positively-selected CD4 and CD8 T cells (using Miltenyi antibodies and isolation column) were used fresh or cryopreserved in 1% human serum albumin (Gemini), 49% plasmalyte (Baxter), and 50% CS10 (Sigma). Cryopreserved cells were thawed, washed in TexMACS (Miltenyi) + 10% human AB serum (Valley Biomedical), and seeded at a density of 2 x 106 cells per mL in TexMACS + 3% human AB serum (culture medium). One day after thaw, or immediately if used fresh, the cells were washed and re-seeded at a density of 1.46 x 106 cells per mL in culture medium + 12.5 ng/mL IL7 + 12.5 ng/mL IL15 + 1:17.5 ratio of TransACT T cell activation reagent (all reagents from Miltenyi) by volume. Two days after activation, T cells were electroporated with i) a plasmid for the production of a NeoTCR Product (*see, e.g.,* **Figure 1B)** or ii) a CD8 Construct 1 (*e.g.,* consisting of the coding sequence of CD8α flanked by P2A sites upstream of the neoTCR beta and alpha sequences and gRNA-Cas9 RNPs targeting the TCR alpha and beta loci; *see, e.g.,* **Figure 2A**). An exemplary expression construct of CD8 Construct 1 is shown in **Figure 11A**. T cells were electroporated using the Lonza X-unit in 100 µL cuvettes and program EO-115. T cells are expanded in culture medium supplemented with 12.5 ng/mL IL7 + 12.5 ng/mL IL15. Supplemented medium was exchanged every 2-3 days until the end of study, 13 days after activation.

comPACT and comPACT-Dextramer preparation. Neoantigen-specific peptide-HLA complex polypeptides (each a "comPACT") were prepared according to the method as described in PCT/US2019/025415, hereby incorporated by reference in its entirety. A comPACT-dextramer complex was made for the labeling of neoTCR expressing T cells. Biotinylated comPACT protein was incubated with a streptavidin-conjugated fluorophore for 10 min at room temperature (RT). Biotin-40-dextran (NANOCS) was added to the mixture and incubated at RT for an additional 10 minutes. The comPACT-Dextramer was stored at 4°C.

Confirmation of comPACT binding to neoTCR edited T cells. T cells were stained for flow cytometry. Cells were first stained with viability dye for 20 minutes at 4°C, then washed and stained with the comPACT-dextramer for 10 minutes at 4°C. Surface antibodies (anti-CD8α, anti-CD8β, anti-CD4) were added to the suspension of cells and comPACT-dextramer, and the cells are incubated for an additional 20 minutes at 4°C. Cells

were then washed and fixed in intracellular fixation buffer (BD Biosciences). All cells were acquired on an Attune NxT Flow Cytometer (ThermoFisher Scientific) and data analyzed with either FCS Express or FlowJo.

Cytometric Bead Array (CBA). Streptavidin coated plates (Eagle Biosciences) were washed 3 times with wash buffer (PBS supplemented with 1% BSA and 0.05% tween20) and then coated with comPACTs at different concentrations ranging from 100-0.01 ng/well. Wells with no comPACT and wells coated with mismatched comPACT were used as controls. The plates were incubated for 2 hr at room temperature, washed three times with wash buffer, and then washed three times with TexMACS supplemented with 3% human AB serum to remove the tween20. T cells were given two washes with TexMACS supplemented with 3% human AB serum and resuspended at 1 million cells/mL in TexMACS supplemented with 3% human AB serum and 1X penicillin-streptomycin solution. T cells were plated onto the comPACT coated plate at 100 µL/well and incubated at 37°C, 5% CO2. After 24h the supernatant was collected, and the cytokine concentrations were analyzed using the BD Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II (Catalog No. 551809) following the manufacturer's protocol. Capture beads were mixed with culture supernatant, incubated with the detection reagent for 3 hr at RT protected from light, washed, and resuspended in wash buffer. Samples were assayed on an Attune NxT Flow Cytometer and data analyzed with FlowJo. The EC50 represents the concentration of cognate comPACT that elicits 50% of the maximum response and is calculated utilizing a least-squares fit of IFNγ secretion over a range of comPACT concentrations.

Intracellular Staining. T cells were stained for flow cytometry on the indicated days. T cells are first stained with viability dye for 20 minutes at 4°C, then washed and incubated with surface antibodies (anti-CD8α, anti-CD8β, anti-CD4) for an additional 20 minutes at 4°C. T cells are then washed and permeabilized for intracellular staining. T cells are stained with anti-2A peptide or with anti-IFNγ, anti-TNF, or anti-IL2 in permeabilization buffer for 20 minutes at 4°C. T cells are fixed in intracellular fixation buffer (BD Biosciences). Samples are assayed on an Attune NxT Flow Cytometer (ThermoFisher Scientific) and data analyzed with either FCS Express or FlowJo.

T cell Proliferation Assay. Edited CD4 and CD8 T cells are labeled with the e450 proliferation dye (eBioscience) according to the manufacturer's instructions. Labeled cells were stimulated on comPACT coated plates with a range of concentrations as described

above. T cells were harvested over 48-96 hours and analyzed for proliferation as measured by dilution of the e450 dye.

T cell Killing Assay. HLA-matched cell lines were pulsed with the cognate neoantigen peptide or mismatched peptide for 1h at 37°C, 5% CO2. The cells were washed 3 times with media to remove any unbound peptide and then co-cultured with edited CD4 and CD8 T cells that are labeled with the e450 proliferation dye described above. Co-cultures were incubated for 48h at 37°C with 5% CO2 before harvest. Cells were washed and stained with a fixable viability dye to determine killing efficiency. The e450 proliferation dye was used to distinguish edited T cells from target cells.

Generation and Validation of CD8 Product 1. An expression construct consisting of the coding sequence (CDS) of CD8α flanked by P2A sites upstream of the neoTCR beta and alpha sequences is synthesized. Briefly, the CDS of human CD8α is synthesized with a GSG-linker and P2A site upstream and flanked with restriction sites. The neoTCR expression vector of interest and the synthesized CD8α construct were incubated with restriction enzymes and ligated together to create the final HDR construct. The CD8 Construct 1 was electroporated along with gRNA-Cas9 RNPs targeting the TCRα and β loci. Model neoTCRs known to bind dextramer among CD8 T cells but not CD4 T cells (*e.g.* TCR097) were used to demonstrate that expression of the CD8α transgene enables these TCRs to bind dextramer among CD4 T cells.

To test the efficiency of the gene transfer and expression of CD8α on the surface of CD4 T cells, engineered CD4 T cells were stained with anti-CD8α antibodies and surface expression of the transgene is confirmed by flow cytometry as described above. NeoTCRs known to bind dextramer among CD8 T cells only (*e.g.,* TCR 097) were used to demonstrate that expression of the CD8α transgene enables these TCRs to bind dextramer among CD4 T cells. *See*, **Figures 13A**, **13B**, **14**, **15A**, and **15B**. Furthermore, CD8α expression on wild type and genetically engineered CD8α T cells was assessed to determine whether the addition of the CD8α transgene increased surface levels of CD8α on CD8 T cells. CD4 T cells engineered to express the CD8α transgene were double-positive for CD4 and CD8α.

CD8 T cells were also engineered to express the CD8α transgene and characterized as described above. **Figures 15A** and **15B**. Relative CD8α gene expression was also quantified via RT-qPCR and compared to control non-engineered CD8 T cells. CD8 T cells expressing the CD8α transgene had higher than endogenous levels of CD8α expression.

Effect of CD8 expression on T cell proliferation upon encounter of cognate antigen. Edited T cells were stained with a proliferation dye as described above. After staining, T cells were stimulated with a range of concentrations of cognate comPACT proteins. 48-72 hours later, T cells were harvested and stained with anti-CD4, anti-CD8, and anti-2A peptide as described above. Edited T cells were identified by 2A expression. Proliferation of the CD4 and CD8 T cells was determined by quantifying the dilution of the proliferative dye. NeoTCR-expressing T cells lacking the CD8α transgene were used as a negative control. NeoTCR CD4 T cells expressing the CD8α transgene proliferated in response to lower concentrations of cognate comPACT than CD4 T cells lacking CD8α expression.

Effect of CD8α expression on cytokine production upon encounter of cognate antigen. In addition to proliferation, effector cytokine production via intracellular cytokine staining was measured. In this assay, NeoTCR Products and CD8 Product 1 were be stimulated with various concentrations of cognate comPACT for 5 hours in the presence of brefeldin A. After stimulation, T cells were stained with anti-CD4 and anti-CD8α. Cells were be permeabilized and stained with anti-P2A peptide, anti-IFNy, anti-TNF, and anti-IL2. NeoTCR CD4 T cells expressing the CD8α transgene produced effector cytokines in response to lower concentrations of cognate comPACT than neoTCR CD4 T cells lacking CD8α expression.

Effect of CD8α expression on killing activity upon encounter of cognate antigen. To assess effector function, edited CD4 and CD8 T cells were cultured with HLA-matched target cells pulsed with cognate peptide as described above. CD4 and CD8 T cells were edited separately to evaluate the ability of CD4 T cells expressing CD8α to kill target cells. NeoTCR CD4 T cells expressing the CD8α transgene killed a greater fraction of target cells presenting cognate peptide than neoTCR CD4 T cells lacking CD8α expression.

Example 3. Generation of CD8 Product 2

CD4 T cells engineered to express CD8α lack CD8β expression. However, CD8β has a higher affinity for LCK (Irie et al., 1998, J. Immunol, 161(1), 183–191). Therefore, T cells were edited to co-express CD8β with CD8α (i.e., a CD8 Product 2). An additional construct containing CD8β flanked by P2A sites, CD8α flanked by P2A sites, followed by the TRB and TRA alleles as previously described is generated. CD4 and CD8 T cells expressing CD8α and CD8β are evaluated using the same assays described above. An

exemplary expression construct with the CD8α and CD8 β sequences is shown in **Figure 11B**.

CD8β expression was also assessed in the edited CD4 T cells. NeoTCR CD4 T cells expressing the CD8α and CD8β transgenes proliferated in response to lower concentrations of cognate comPACT than CD4 T cells expressing CD8α transgene alone. NeoTCR CD4 T cells expressing the CD8α and CD8β transgenes also produced effector cytokines in response to lower concentrations of cognate comPACT than neoTCR CD4 T cells expressing the CD8α transgene alone. And neoTCR CD4 T cells expressing the CD8α and CD8β transgenes killed a greater fraction of target cells presenting cognate peptide than neoTCR CD4 T cells expressing CD8α transgene alone.

Example 4. Generation of chimeric CD8α and CD8β Constructs and CD8 Products 3 and 4

To ensure efficient editing of the T cells and expression of the neoTCR, chimeric proteins made up of the coding sequences for the extracellular and transmembrane domains of CD8α linked to the intracellular domain of CD8β were generated. An exemplary expression construct with the extracellular and transmembrane domains of CD8α linked to the intracellular domain of CD8β sequences is (*i.e.,* a CD8 Product 3) shown in **Figure 11C**.

Furthermore, the intracellular domain of CD4 has an even higher affinity for LCK than CD8 (Irie et al., 1998). Therefore, a second chimeric protein was generated containing the coding sequences for the extracellular and transmembrane domains of CD8α linked to the intracellular domain of CD4. An exemplary expression construct with the extracellular and transmembrane domains of CD8α linked to the intracellular domain of CD4 sequences (*i.e.,* a CD8 Product 4) is shown in **Figure 11D**.

CD4 and CD8 T cells expressing the CD8 Products 3 and 4 were evaluated using the same assays described above.

NeoTCR CD4 T cells expressing the CD8α-CD8β-ID transgene proliferated in response to lower concentrations of cognate comPACT than CD4 T cells expressing CD8α transgene alone. NeoTCR CD4 T cells expressing the CD8α-CD8β-ID transgene produced effector cytokines in response to lower concentrations of cognate comPACT than neoTCR CD4 T cells expressing the CD8α transgene alone. NeoTCR CD4 T cells expressing the

CD8α-CD8β-ID transgene killed a greater fraction of target cells presenting cognate peptide than neoTCR CD4 T cells expressing CD8α-CD8β-ID transgene.

NeoTCR CD4 T cells expressing the CD8α-CD4-ID transgene proliferated in response to lower concentrations of cognate comPACT than neoTCR CD4 T cells expressing CD8α-CD8β-ID transgene. NeoTCR CD4 T cells expressing the CD8α-CD4 transgene produced effector cytokines in response to lower concentrations of cognate comPACT than neoTCR CD4 T cells expressing the CD8α-CD8β-ID transgene.

NeoTCR CD4 T cells expressing the CD8α-CD4-ID transgene killed a greater fraction of target cells presenting cognate peptide than neoTCR CD4 T cells expressing CD8α-CD8β-ID transgene. NeoTCR CD8 T cells expressing the CD8α-CD4-ID transgene proliferated in response to lower concentrations of cognate comPACT than neoTCR CD8 T cells lacking the transgene. NeoTCR CD8 T cells expressing the CD8α-CD4 transgene produced effector cytokines in response to lower concentrations of cognate comPACT than neoTCR CD8 T cells lacking the transgene. NeoTCR CD8 T cells expressing the CD8α-CD4-ID transgene killed a greater fraction of target cells presenting cognate peptide than neoTCR CD8 T cells lacking the transgene.

Example 5. CD8 Products have increased sensitivity to neoE-HLA target recognition and trigger pro-inflammatory and cytotoxic function

MHC-I neoTCRs were cloned from neoE-specific T cells captured from the blood of a patient with colorectal cancer. Healthy donor CD8 and CD4 T cells were precision genome engineered to express the cloned MHC-I neoTCRs alone or to include engineering of ectopic CD8 co-receptors in the gene-edited T cells. Flow cytometric analysis was used to evaluate surface expression of neoTCRs and ectopic CD8 co-receptors (i.e., the CD8 and CD4 components of the CD8 Constructs 1-4), respectively. Rescue of neoTCR binding among CD4 T cells for lower affinity, CD8-dependent neoTCRs was observed. Importantly, in response to stimulation with cognate antigen, CD107a and intracellular IFNy staining revealed 10-100-fold increases in the sensitivity of MHC-I neoTCR-induced effector functions by CD4 T cells, with no effect on specificity. No change in functionality or sensitivity was seen on CD8 T cells by the expression of additional CD8 co-receptor.

These results demonstrate that simultaneous precision genome engineering of the CD8 co-receptor together with CD8-dependent MHC-I neoTCRs into CD4 T cells (*i.e.*, CD8 Products 1-4) significantly increases their sensitivity to neoE-HLA target recognition

as well as triggering pro-inflammatory and cytotoxic function, yet without compromising antigen-specificity.

Example 6. Generation and design of CD8 Products that have varying LCK affinities

As described herein, four classes of CD8 Products were generated:

1. CD8α homodimer (CD8 Construct 1)
2. CD8α-P2A-CD8β (CD8 Construct 2)
3. CD8α with CD8β intracellular domain (CD8 Construct 3)
4. CD8α homodimer with CD4 intracellular domain (CD8 Construct 4)

As shown in **Figure 10**, these CD8 Constructs and resulting CD8 Products were designed to allow for varying degrees of LCK affinity. As predicted, CD8 Product 1 was shown to have the lowest LCK affinity, followed by CD8 Product 2, CD8 Product 3, and CD8 Product 4 (in that order with CD8 Product 4 having the highest LCK affinity.

Based on the high affinity of CD8 Product 4, this product was used in cell killing assays to exemplify the increased cell killing ability of CD8 Products 1-4 compared to NeoTCR Products.

CD4+ T cells were engineered as described herein to express the CD8 Product 4 (with TCR097 as the NeoTCR in the product) described in **Figured 2D** and **3D**. SW620 cell lines that were engineered to heterologously express the R20Q mutation (the cognate antigen to TCR097). The CD8 Product 4 expressing NeoTCR097 was combined with the SW620 heterologous cells. As shown in **Figures 13A** and **13B**, the CD8 Product 4 provided substantially better killing of the cognate antigen expressing SW620 cells than the NeoTCR Product also expressing the TCR097. The experiment shown in **Figure 13A** was done with an E:T ratio of 1:1 and as shown in the graph, the NeoTCR Product expressing TCR097 did not show any efficacy at killing of the cognate antigen expressing SW620 cells. The experiment shown in **Figure 13B** was done with an E:T ratio of 2:1 and while the NeoTCR Product expressing TCR097 showed some ability to kill the cognate antigen expressing SW620 cells, it was clear from the experiment that the CD8 Product 4 expressing the TCR097 had superior efficacy.

The same engineered CD4+ cells that were used in the experiment above with data provided in **Figures 13A** and **13B**, were also tested on SW620 cell lines that were engineered to homozygously express the R20Q mutation. In this experiment, the high

expression of the cognate antigen was able to compensate for the low affinity NeoTCR097 and both the NeoTCR Product expressing TCR097 and the CD8 Product 4 expressing the TCR097 showed efficacy at killing the SW620 cells. However, the high expression of the cognate antigen in the homozygous SW620 cells is not physiologically relevant and this experiment serves to highlight the ability to rescue NeoTCR Products with low affinity TCRs that cannot effectively engage with and kill tumor cells by further engineering them to also include a CD8α homodimer with CD4 intracellular domain (*i.e.*, a CD8 Product 4).

As a final control and proof of the efficacy of the CD8 Products, CD8 T cells were also transfected to express the NeoTCR097 and a CD8α homodimer with CD4 intracellular domain (*i.e.*, a CD8 Product 4 using CD8 T cells instead of CD4 T cells). As shown in the top graphs in **Figures 15A** and **15B**, the CD8 Product 4 provided substantially better killing of the SW620 cells than the NeoTCR Product when the products were made from CD4 T cells. However, when the CD8 Product 4 and NeoTCR Product were made from CD8 T cells (the bottom graphs in **Figures 15A** and **15B**) the ability of the NeoTCR Product was rescued because of the endogenous CD8 expression in the CD8 T cells. While no overlay of the graphs is shown, it is also of note that the CD8 Product 4 in CD4 T cells shown the top graphs in **Figures 15A** and **15B**, appear to have better efficacy than the CD8 Product 4 in CD8 T cells in the bottom two graphs; suggesting a superior ability of the CD8 Products described herein to engage with the cognate antigens of tumor cells leading to tumor death and effective treatment of patients with cancer in need of treatment.

Example 6. CD8 Products have increased CD4 T cell sensitivity while maintaining NeoTCR sensitivity

In order to confirm that the CD8 Constructs expressed properly, CD8 Products 1, 2, 3, and 4 were tested to determine surface expression of CD8α. It was shown that each of CD8 Products 1, 2, 3, and 4 exhibited normal CD8α surface expression. Representative data from CD8 Product 4 is shown in **Figure 16**.

It was also important to determine how the CD8 Constructs affected CD4 T cell sensitivity and specificity for the cognate antigen of the expressed NeoTCR in the CD8 Products. Sensitivity experiments were performed and it was shown that CD8 Products 1-4 exhibited an increased CD4 T cell sensitivity (**Figure 17A**). Specificity experiments were also performed on CD8 Products 1-4. CD8 Products 1, 2, 3, and 4 were made with NeoTCR097. Experiments were performed to test the CD8 Products 1, 2, 3, and 4

(expressing NeoTCR097) to assess the specificity of these products to the cognate antigen to NeoTCR097. As shown in **Figure 17B**, CD8 Products 1, 2, 3, and 4 (expressing NeoTCR097) were specific for the cognate antigen to NeoTCR097 and showed no activity when exposed to a mismatched antigen. The specificity was determined by INFγ and CD107 production which are evidence of T cell activation. Thus, the CD8 Products described herein have an increased sensitivity to CD4 T cells and maintain their specificity to cognate antigen to the expressed NeoTCR compared to NeoTCR Products expressing the same NeoTCR.

Lastly, experiments were performed to investigate the impact the CD8 Constructs have on CD8-dependent and CD8-independent NeoTCRs. It was expected that CD8-dependent NeoTCRs would show an increased sensitivity to cognate antigen because the CD8 Cells would be engineered to express CD8α and that CD8-independent NeoTCRs would not show an increased sensitivity because of the independent nature of the NeoTCR. However, it was shown that NeoTCR Products 1, 2, 3, and 4 surprisingly increased the sensitivity of CD8-dependent (e.g., NeoTCR097) and CD8-independent (e.g., NeoTCR089) NeoTCRs. Accordingly, it was shown that the CD8 Constructs 1, 2, 3, and 4 can improve NeoTCR engagement and T cell killing of tumor cells with cognate NeoTCR antigens for all NeoTCRs regardless of whether they are CD8-dependent or CD8-independent.

While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

WHAT IS CLAIMED IS:

1. A cell, comprising:
    a. an exogenous T cell receptor (TCR); and
    b. an exogenous CD8.
2. The cell of claim 1, wherein the exogenous CD8 comprises at least one monomer.
3. The cell of claim 2, wherein the at least one monomer of the exogenous CD8 comprises an extracellular domain, a transmembrane domain, an intracellular domain, fragments thereof, or combinations thereof.
4. The cell of claim 3, wherein the extracellular domain comprises a CD8α extracellular domain or a CD8β extracellular domain.
5. The cell of claim 3 or 4, wherein the transmembrane a CD8α transmembrane domain or a CD8β transmembrane domain.
6. The cell of any one of claims 3-5, wherein the intracellular domain comprises a CD8α intracellular domain or a CD8β intracellular domain.
7. The cell of any one of claims 3-5, wherein the intracellular domain comprises a CD4 intracellular domain.
8. The cell of any one of claims 2-6, wherein the at least one monomer comprises a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain.
9. The cell of any one of claims 2-6, wherein the at least one monomer comprises a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain.
10. The cell of any one of claims 2-6, wherein the at least one monomer comprises a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain.
11. The cell of any one of claims 2-7, wherein the at least one monomer comprises a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.
12. The cell of any one of claims 2-11, wherein the at least one monomer comprises a signal peptide.
13. The cell of claim 12, wherein the signal peptide is a CD8 signal peptide.
14. The cell of any one of claims 3-13, wherein the extracellular domain comprises the amino acid sequence set forth in SEQ ID NO: 140, or SEQ ID NO: 145.

15. The cell of any one of claims 3-14, wherein the transmembrane domain comprises the amino acid sequence set forth in SEQ ID NO: 141, or SEQ ID NO: 146.
16. The cell of any one of claims 3-15, wherein the intracellular domain comprises the amino acid sequence set forth in SEQ ID NO: 142, SEQ ID NO: 147, or SEQ ID NO: 148.
17. The cell of any one of claims 12-16, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO: 139, or SEQ ID NO: 144.
18. The cell of any one of claims 1-17, wherein the exogenous CD8 comprises a 2A sequence.
19. The cell of any one of claims 1-18, wherein the exogenous CD8 comprises a linker.
20. The cell of claim 19, wherein the linker comprises the amino acid sequence set forth in SEQ ID NO: 137.
21. The cell of any one of claims 1-20, wherein the exogenous CD8 comprises a protease cleavage site.
22. The cell of claim 21, wherein the protease cleavage site is a Furin cleavage site.
23. The cell of any one of claims 1-22, wherein the exogenous TCR is a patient derived TCR.
24. The cell of any one of claims 1-23, wherein the exogenous TCR comprises a signal sequence, a first and second 2A sequence, and a TCR polypeptide sequence.
25. The cell of any one of claims 1-24, wherein the exogenous TCR recognizes a cancer antigen.
26. The cell of claim 25, wherein the cancer antigen is a neoantigen.
27. The cell of claim 25, wherein the cancer antigen is a patient specific antigen.
28. The cell of any one of claims 1-27, wherein the cell is a primary cell.
29. The cell of any one of claims 1-27, wherein the cell is a patient-derived cell.
30. The cell of any one of claims 1-27, wherein the cell is a lymphocyte.
31. The cell of any one of claims 1-27, wherein the cell is a T cell.
32. The cell of any one of claims 1-27, wherein the cell if a young T cell.
33. The cell of claim 32, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.
34. The cell of claim 32, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.
35. The cell of claim 32, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

36. The cell of any one of claims 1-35, further comprising a gene modification to enhance cell persistence and/or enhances memory cell differentiation
37. The cell of any one of claims 1-36, wherein killing activity of the cell is increased between about 10% to about 500% as compared to killing activity of a cell that does not have the exogenous CD8.
38. The cell of any one of claims 1-37, wherein proliferation of the cell upon binding of the TCR to the antigen is increased between about 10% to about 500% as compared to proliferation of a cell that does not have the exogenous CD8.
39. The cell of any one of claims 1-38, wherein secretion of pro-inflammatory cytokine upon binding of the TCR to the antigen by the cell is increased between about 10% to about 500% as compared to secretion by a cell that does not have the exogenous CD8.
40. The cell of any one of claims 1-39, wherein LCK affinity of the cell is increased between about 10% to about 500% as compared to LCK affinity of a cell that does not have the exogenous CD8.
41. The cell of any one of claims 1-40, wherein persistence of the cell is increased between about 10% to about 500% as compared to persistence of a cell that does not have the exogenous CD8.
42. The cell of any one of claims 1-41, wherein tumor infiltration ability of the cell is increased between about 10% to about 500% as compared to tumor infiltration ability of a cell that does not have the exogenous CD8.
43. The cell of any one of claims 1-42, wherein the exogenous TCR is a CD8-dependent TCR.
44. The cell of any one of claims 1-42, wherein the exogenous TCR is a CD8-independent TCR.
45. The cell of any one of claims 1-44, wherein the exogenous CD8 is encoded by a CD8 Construct 1, a CD8 Construct 2, a CD8 Construct 3, or a CD8 Construct 4.
46. The cell of any one of claims 1-44, wherein the exogenous CD8 comprises:
    a. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain;
    b. a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain;

c. a CD8α extracellular domain, a CD8α transmembrane domain, and aCD8β intracellular domain; or

d. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

47. The cell of any one of claims 1-44, wherein the exogenous CD8 comprises:

a. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142);

b. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147);

c. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or

d. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

48. A method of modifying a cell, the method comprising:

a. introducing into the cell a homologous recombination (HR) template nucleic acid sequence, wherein the HR template comprises:

i. first and second homology arms homologous to first and second target nucleic acid sequences;

ii. a TCR gene sequence positioned between the first and second homology arms;

iii. a CD8 gene sequence positioned between the first and the second homology arms; and

b. recombining the HR template nucleic acid into an endogenous locus of the cell.

49. The method of claim 48, wherein the HR template comprises a first 2A-coding sequence positioned upstream of the CD8 gene sequence, a second 2A-coding sequence positioned downstream of the CD8 gene sequence and upstream of the

TCR gene sequence, and a third 2A-coding sequence positioned downstream of the TCR gene sequence; wherein the first, second, and third 2A-coding sequences code for the same amino acid sequence and are codon-diverged relative to each other.

50. The method of claim 48 or 49, wherein the HR template comprises a sequence coding for the amino acid sequence Gly Ser Gly positioned immediately upstream of the first, second, and/or third 2A-coding sequences.

51. The method of claim 49 or 50, wherein the HR template further comprises a sequence coding for a Furin cleavage site positioned upstream of the first, second, and/or third 2A-coding sequences.

52. The method of any one of claims 48-51, wherein the HR template further comprises a sequence encoding a signal sequence positioned immediately upstream of the TCR gene sequence and/or the CD8 gene sequence.

53. The method of any one of claims 48-52, wherein the HR template comprises a second TCR sequence positioned between the third 2A-coding sequence and the second homology arm.

54. The method of claim 53, wherein the HR template comprises:

    a. a sequence encoding a first signal sequence positioned immediately upstream the first TCR gene sequence; and

    b. a sequence encoding a second signal sequence positioned immediately upstream of the second TCR gene sequence.

55. The method of any one of claims 48-54, wherein the HR template comprises a second CD8 gene sequence positioned between the first CD8 gene sequence and the second 2A-coding sequence.

56. The method of claims 55, wherein a 2A coding sequence is positioned between the first and second CD8 gene sequence.

57. The method of claim 55 or 56, wherein a sequence coding for the amino acid sequence Gly Ser Gly is positioned between the first and second CD8 gene sequences.

58. The method of any one of claims 55-57, wherein a sequence coding for a Furin cleavage site is positioned between the first and second CD8 gene sequences.

59. The method of any one of claims 48-58, wherein the CD8 gene sequence comprises a sequence encoding an extracellular domain, a sequence encoding an intracellular domain, a sequence encoding an intracellular domain, fragments thereof, or combinations thereof.

60. The method of claim 59, wherein the sequence encoding an extracellular domain comprises a sequence encoding a CD8α extracellular domain or a CD8β extracellular domain.
61. The method of claim 59 or 60, wherein the sequence encoding a transmembrane domain comprises a sequence encoding a CD8α transmembrane domain or a CD8β transmembrane domain.
62. The method of any one of claims 59-61, wherein the sequence encoding an intracellular domain comprises a sequence encoding a CD8α intracellular domain or a CD8β intracellular domain.
63. The method of any one of claims 59-61, wherein the sequence encoding an intracellular domain comprises a sequence encoding a CD4 intracellular domain.
64. The method of any one of claims 59-62, wherein the CD8 gene sequence comprises a sequence encoding a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain.
65. The method of any one of claims 59-62, wherein the CD8 gene sequence comprises a sequence encoding a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain.
66. The method of any one of claims 59-62, wherein the CD8 gene sequence comprises a sequence encoding a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain.
67. The method of any one of claims 59-63, wherein the CD8 gene sequence comprises a sequence encoding a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.
68. The method of any one of claims 55-67, wherein the HR template comprises:
    a. a sequence encoding a first signal sequence positioned immediately upstream the first CD8 gene sequence; and
    b. a sequence encoding a second signal sequence positioned immediately upstream of the second CD8 gene sequence.
69. The method of any one of claims 52-68, wherein the signal sequence is a CD8 signal sequence, a human growth hormone signal sequence, fragments thereof, or combinations thereof.
70. The method of any one of claims 48-69, wherein the first and second homology arms of the HR template are each from about 300 bases to about 2,000 bases in length.

71. The method of any one of claims 48-70, wherein the first and second homology arms of the HR template are each from about 600 bases to about 2,000 bases in length.
72. The method of any one of claims 48-71, wherein the exogenous TCR is a patient derived TCR.
73. The method of any one of claims 48-72, wherein the exogenous TCR comprises a signal sequence, a first and second 2A sequence, and a TCR polypeptide sequence.
74. The method of any one of claims 48-73, wherein the exogenous TCR recognizes a cancer antigen.
75. The method of claim 74, wherein the cancer antigen is a neoantigen.
76. The method of claim 74, wherein the cancer antigen is a patient specific antigen.
77. The method of any one of claims 48-76, wherein the HR template is non-viral.
78. The method of any one of claims 48-77, wherein the HR template is a circular DNA.
79. The method of any one of claims 48-77, wherein the HR template is a linear DNA.
80. The method of any one of claims 48-79, wherein the introducing occurs via electroporation.
81. The method of any one of claims 48-80, wherein the recombining comprises:
    a. cleavage of the endogenous locus by a nuclease; and
    b. recombination of the HR template nucleic acid sequence into the endogenous locus by homology directed repair.
82. The method of claim 81, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.
83. The method of claim 82, further comprising a gRNA.
84. The method of any one of claims 48-83, wherein the process further comprises culturing the cell.
85. The method of claim 84, wherein the culturing is conducted in the presence of at least one cytokine.
86. The method of claim 84 or 85, wherein the culturing is conducted in the presence of IL2, IL7, IL15, or any combination thereof.
87. The method of claim 84 or 85, wherein the culturing is conducted in the presence of IL7 and IL15.
88. The method of any one of claims 48-87, further comprising a gene modification to enhance cell persistence and/or enhances memory cell differentiation.

89. The method of any one of claims 48-88, wherein the cell is a primary cell.

90. The method of any one of claims 48-88, wherein the cell is a patient-derived cell.

91. The method of any one of claims 48-88, wherein the cell is a lymphocyte.

92. The method of any one of claims 48-88, wherein the cell is a T cell.

93. The method of any one of claims 48-88, wherein the cell is a young T cell.

94. The method of claim 93, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.

95. The method of claim 93, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.

96. The method of claim 93, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

97. The method of any one of claims 48-96, wherein killing activity of the cell is increased between about 10% to about 500% as compared to killing activity of a cell that does not have the CD8 gene sequence.

98. The method of any one of claims 48-97, wherein proliferation of the cell upon binding of the TCR to the antigen is increased between about 10% to about 500% as compared to proliferation of a cell that does not have the CD8 gene sequence.

99. The method of any one of claims 48-98, wherein secretion of pro-inflammatory cytokine upon binding of the TCR to the antigen by the cell is increased between about 10% to about 500% as compared to secretion by a cell that does not have the CD8 gene sequence.

100. The method of any one of claims 48-99, wherein LCK affinity of the cell is increased between about 10% to about 500% as compared to LCK affinity of a cell that does not have the CD8 gene sequence.

101. The method of any one of claims 48-100, wherein persistence of the cell is increased between about 10% to about 500% as compared to persistence of a cell that does not have the CD8 gene sequence.

102. The method of any one of claims 48-101, wherein tumor infiltration ability of the cell is increased between about 10% to about 500% as compared to tumor infiltration ability of a cell that does not have the CD8 gene sequence.

103. The method of any one of claims 48-102, wherein the TCR gene encodes a CD8-dependent TCR.

104. The method of any one of claims 48-102, wherein the TCR gene encodes a CD8-independent TCR.

105. The method of any one of claims 48-104, wherein the CD8 gene sequence is encoded by a CD8 Construct 1, a CD8 Construct 2, a CD8 Construct 3, or a CD8 Construct 4.

106. The method of any one of claims 48-104, wherein the CD8 gene sequence comprises:

a. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain;

b. a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain;

c. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or

d. a CD8α extracellular domain, CD8α transmembrane domain, CD4 intracellular domain.

107. The method of any one of claims 48-104, wherein the CD8 gene sequence comprises:

a. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142);

b. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147);

c. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or

d. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

108. A cell modified by the method of any one of claims 48-107.

109. A composition comprising an effective amount of a cell of any one of claims 1-47 or a cell of claim 108.

110. The composition of claim 109, wherein the composition is a pharmaceutical composition that further comprises a pharmaceutically acceptable excipient.

111. The composition of claim 109 or 110, wherein the composition is administered to a patient in need thereof for the treatment of cancer.

112. The composition of any one of claims 109-111, wherein the composition comprises a cryopreservation agent.

113. The composition of any one of claims 109-112, wherein the composition comprises serum albumin.

114. The composition of any one of claims 109-113, wherein the composition comprises Plasma-Lyte A, HSA, and CryoStor CS10.

115. A method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell of any one of claims 1-47, a cell of claim 108, or a composition of any one of claims 109-114.

116. The method of claim 115, wherein prior to administering the therapeutically effective amount of cells, a non-myeloablative lymphodepletion regimen is administered to the subject.

117. The method of claim 115 or 116, wherein the cancer is a solid tumor.

118. The method of claim 115 or 116, wherein the cancer is liquid tumor.

119. The method of claim 117, wherein the solid tumor is selected from the group consisting of melanoma, thoracic cancer, lung cancer, ovarian cancer, breast cancer, pancreatic cancer, head and neck cancer, prostate cancer, gynecological cancer, central nervous system cancer, cutaneous cancer, HPV+ cancer, esophageal cancer, thyroid cancer, gastric cancer, hepatocellular cancer, cholangiocarcinomas, renal cell cancers, testicular cancer, sarcomas, and colorectal cancer.

120. The method of claim 118, wherein the liquid tumor is selected from the group consisting of follicular lymphoma, leukemia, and multiple myeloma.

121. A kit comprising a cell of any one of claims 1-47, reagents for performing the method of any one of claims 48-107, a cell of claim 108, or a composition of any one of claims 109-114.

122. The kit of claim 121, wherein the kit further comprises written instructions for treating a cancer.

123. A cell, comprising:

a. an exogenous T cell receptor (TCR); and

b. an exogenous CD8, comprising:

i. a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain;

ii. a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain;

iii. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or

iv. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

124. A cell, comprising:

a. an exogenous T cell receptor (TCR); and

b. an exogenous CD8, comprising:

i. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142);

ii. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147);

iii. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or

iv. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

125. A method of modifying a cell, the method comprising:

a. introducing into the cell a homologous recombination (HR) template nucleic acid sequence, wherein the HR template comprises:

i. first and second homology arms homologous to first and second target nucleic acid sequences;

ii. a TCR gene sequence positioned between the first and second homology arms;

iii. a CD8 gene sequence positioned between the first and the second homology arms; and

b. recombining the HR template nucleic acid into an endogenous locus of the cell,

wherein the CD8 gene sequence comprises:

i. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain;

ii. a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain;

iii. a CD8α extracellular domain, a CD8α transmembrane domain, a CD8β intracellular domain; or

iv. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

126. A method of modifying a cell, the method comprising:

a. introducing into the cell a homologous recombination (HR) template nucleic acid sequence, wherein the HR template comprises:

i. first and second homology arms homologous to first and second target nucleic acid sequences;

ii. a TCR gene sequence positioned between the first and second homology arms;

iii. a CD8 gene sequence positioned between the first and the second homology arms; and

b. recombining the HR template nucleic acid into an endogenous locus of the cell,

wherein the CD8 gene sequence comprises:

i. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142);

ii. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a

CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147);

iii. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or

iv. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

127. A composition comprising a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises:

a. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain;

b. a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain,

c. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or

d. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

128. A composition comprising a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises:

a. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142);

b. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147);

c. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or

d. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

129. A method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises:

a. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8α intracellular domain;

b. a CD8α extracellular domain, a CD8α transmembrane domain, a CD8α intracellular domain, a CD8β extracellular domain, a CD8β transmembrane domain, and a CD8β intracellular domain,

c. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD8β intracellular domain; or

d. a CD8α extracellular domain, a CD8α transmembrane domain, and a CD4 intracellular domain.

130. A method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell, wherein the cell comprises an exogenous T cell receptor (TCR) and an exogenous CD8, wherein the exogenous CD8 comprises:

a. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8α intracellular domain (SEQ ID NO: 142);

b. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), a CD8α intracellular domain (SEQ ID NO: 142), a CD8β signal peptide (SEQ ID NO:144), a CD8β extracellular domain (SEQ ID NO:145), a CD8β transmembrane domain (SEQ ID NO:146), and a CD8β intracellular domain (SEQ ID NO: 147);

c. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD8β intracellular domain (SEQ ID NO: 147); or

d. a CD8α signal peptide (SEQ ID NO:139), a CD8α extracellular domain (SEQ ID NO:140), a CD8α transmembrane domain (SEQ ID NO:141), and a CD4 intracellular domain (SEQ ID NO: 148).

FIGURE 1A

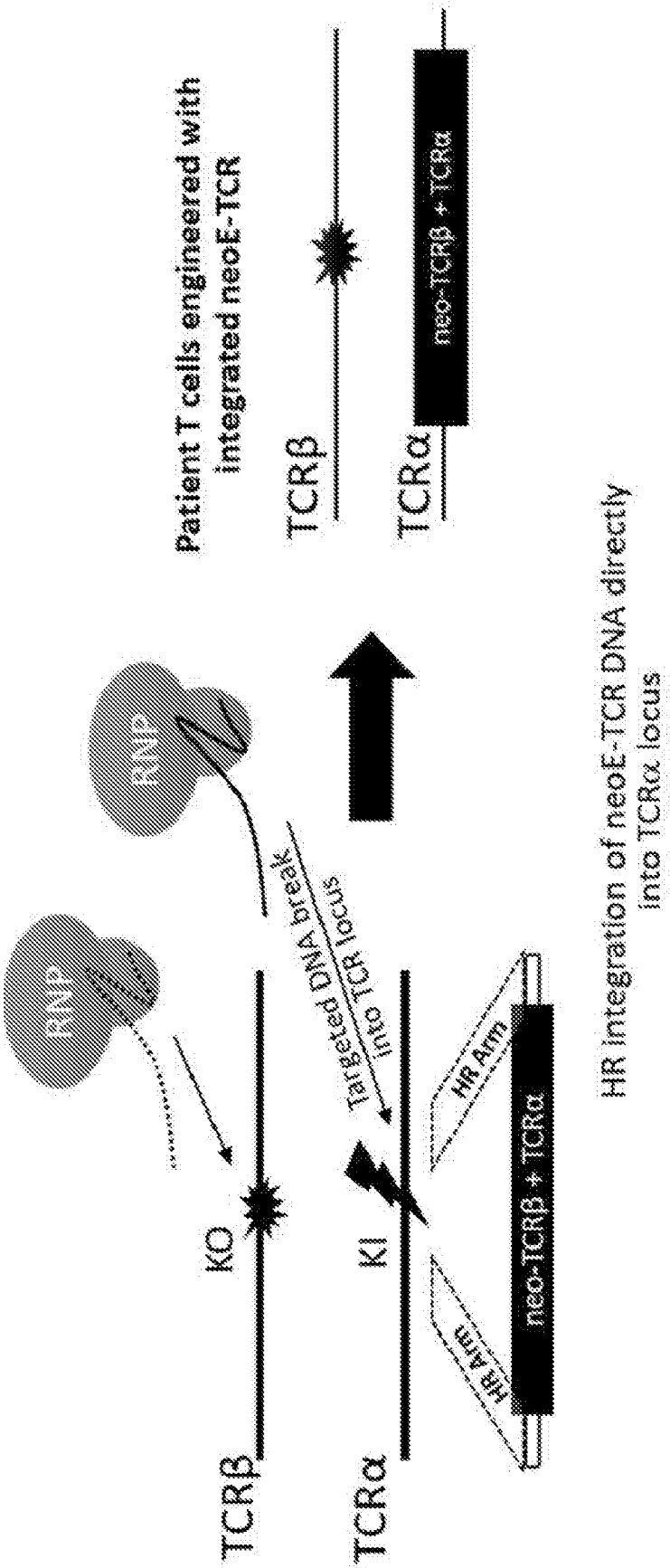

Patient T cells engineered with integrated neoE-TCR
TCRβ
TCRα
neo-TCRβ + TCRα
RNP
RNP
Targeted DNA break into TCR locus
KO
KI
HR Arm
HR Arm
neo-TCRβ + TCRα
TCRβ
TCRα
HR integration of neoE-TCR DNA directly into TCRα locus

FIGURE 1B

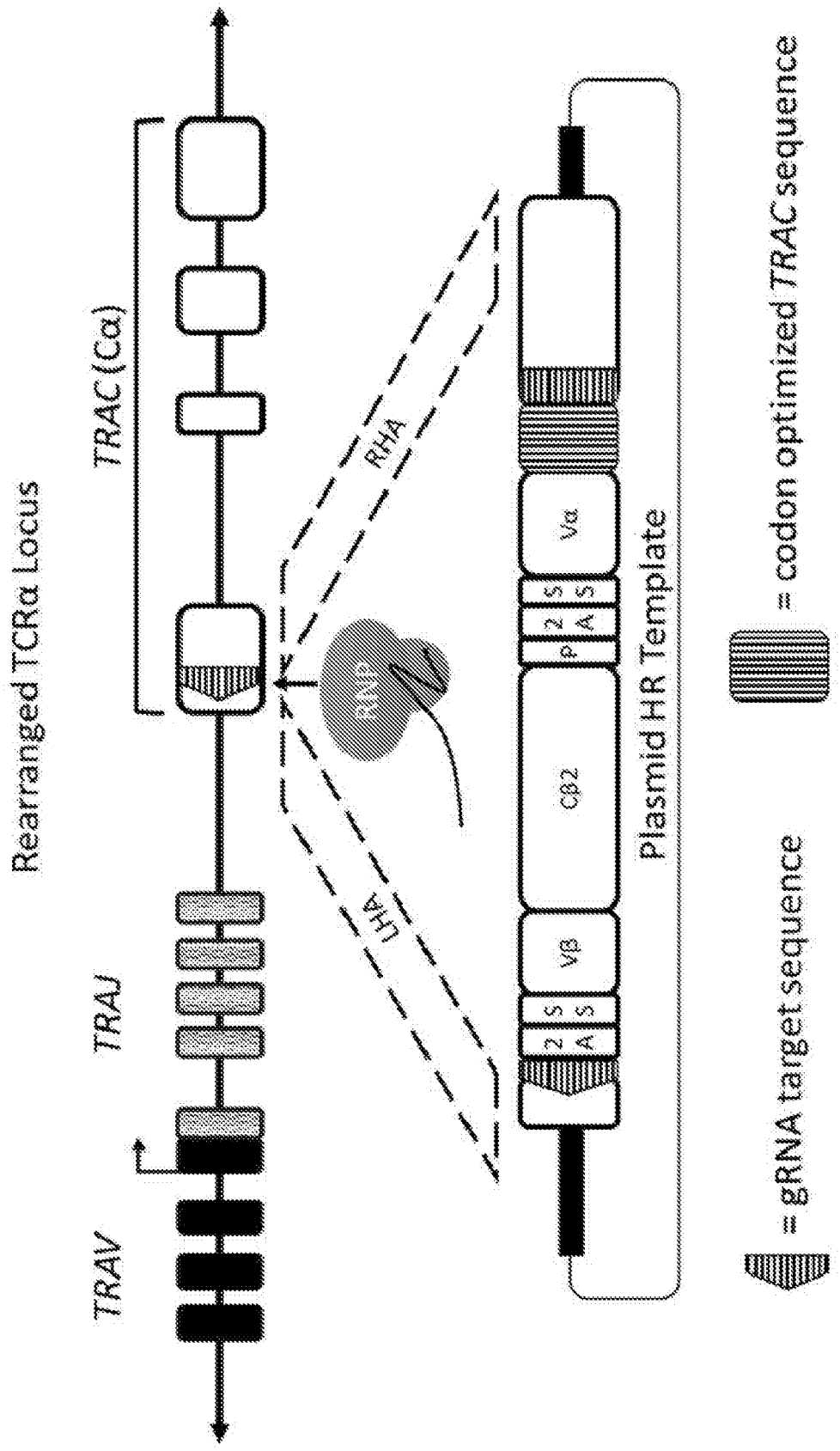

Rearranged TCRα Locus
TRAV
TRAJ
TRAC (Cα)
RHA
LHA
gRNA
Plasmid HR Template
Vα
S
S
2
A
P
Cβ2
Vβ
S
S
2
A
= codon optimized TRAC sequence
= gRNA target sequence

FIGURE 1C

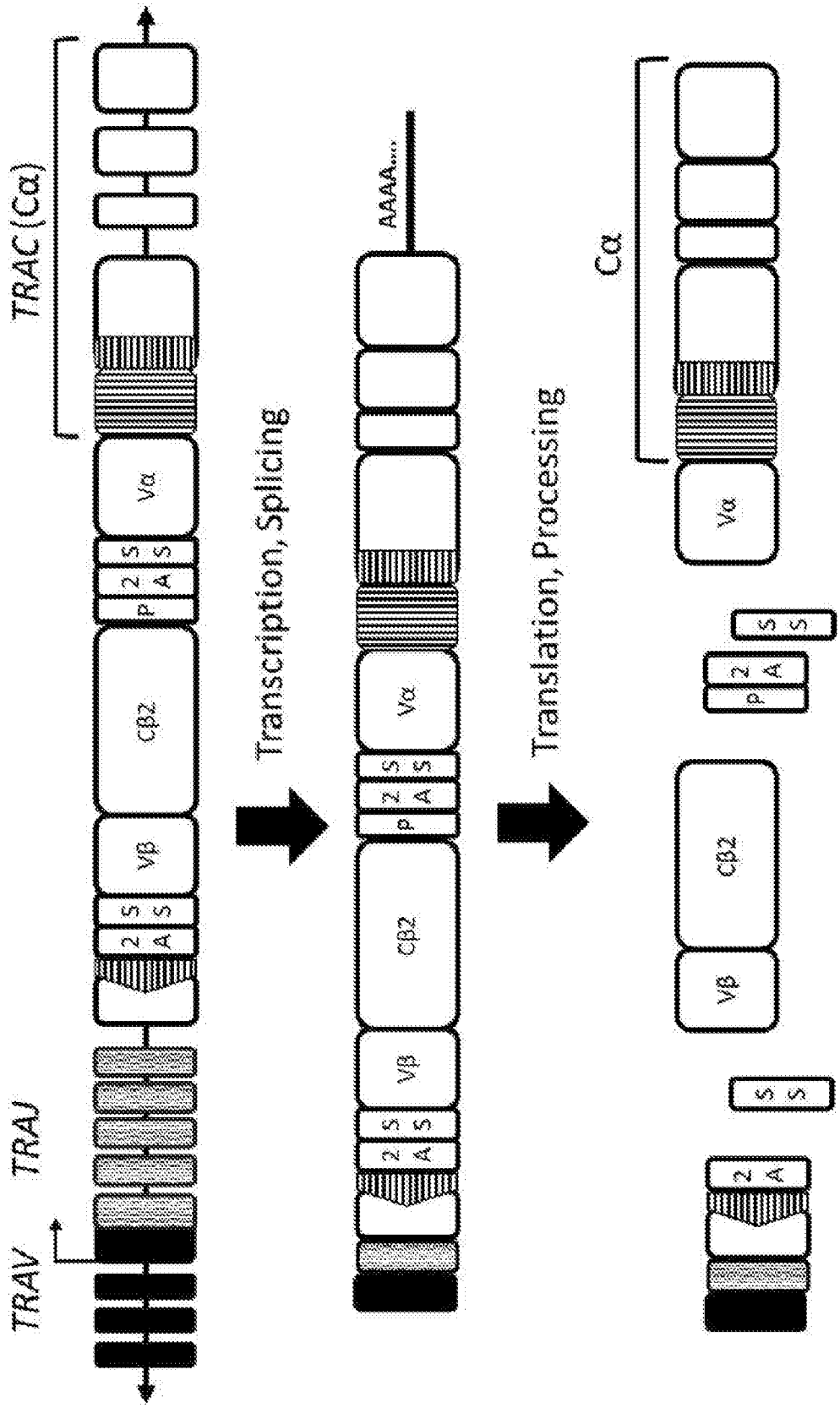

TRAC (Cα)
TRAV
TRAJ
2
A
S
S
Vβ
Cβ2
P
2
S
A
S
Vα
Transcription, Splicing
AAAA....
Translation, Processing
Cα

FIGURE 2A

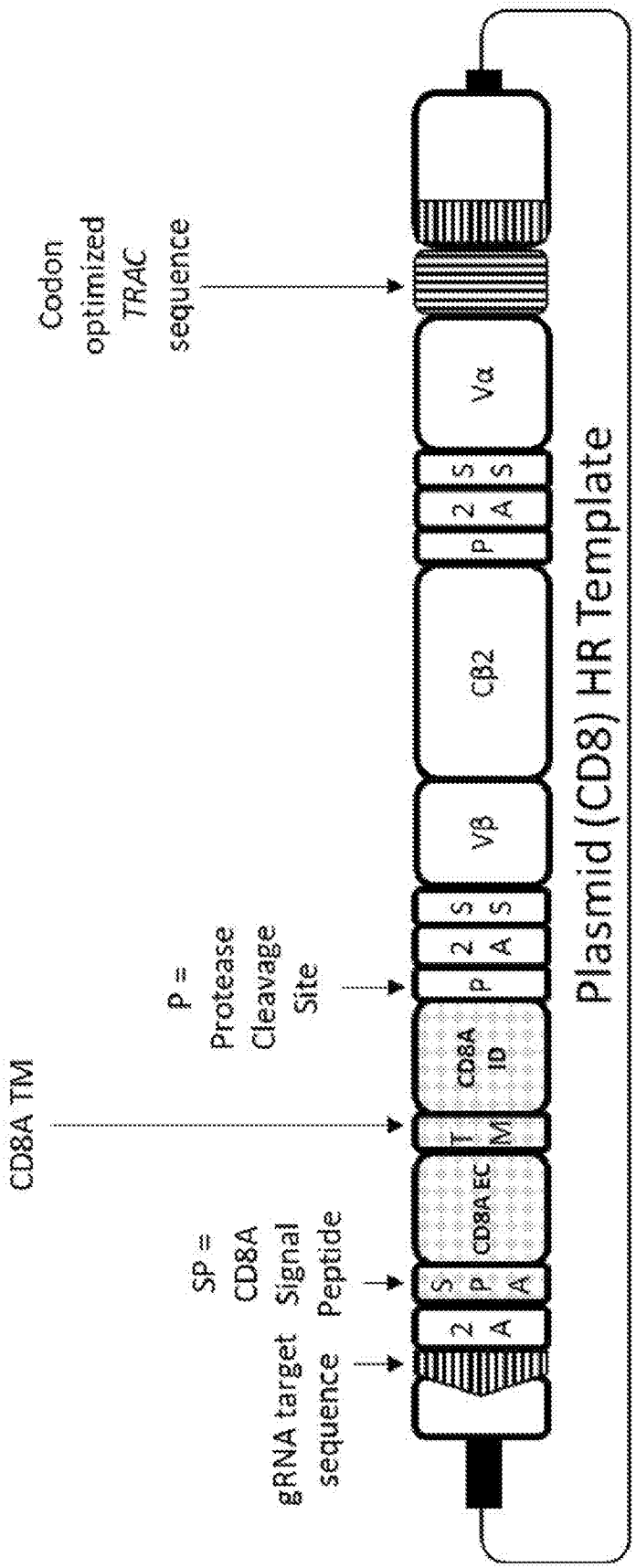

Codon optimized TRAC sequence
CD8A TM
P = Protease Cleavage Site
SP = CD8A Signal Peptide
gRNA target sequence
Vα
Cβ2
Vβ
CD8A EC
CD8A ID
Plasmid (CD8) HR Template
CD8 Product 1

FIGURE 2B

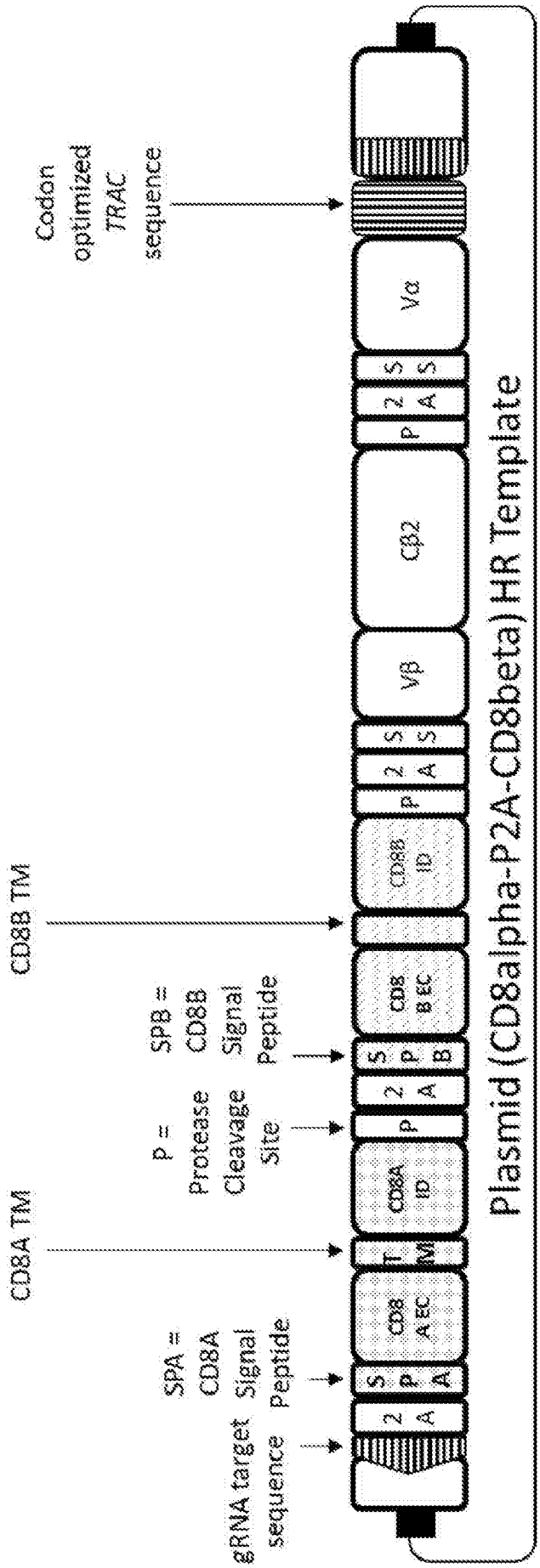
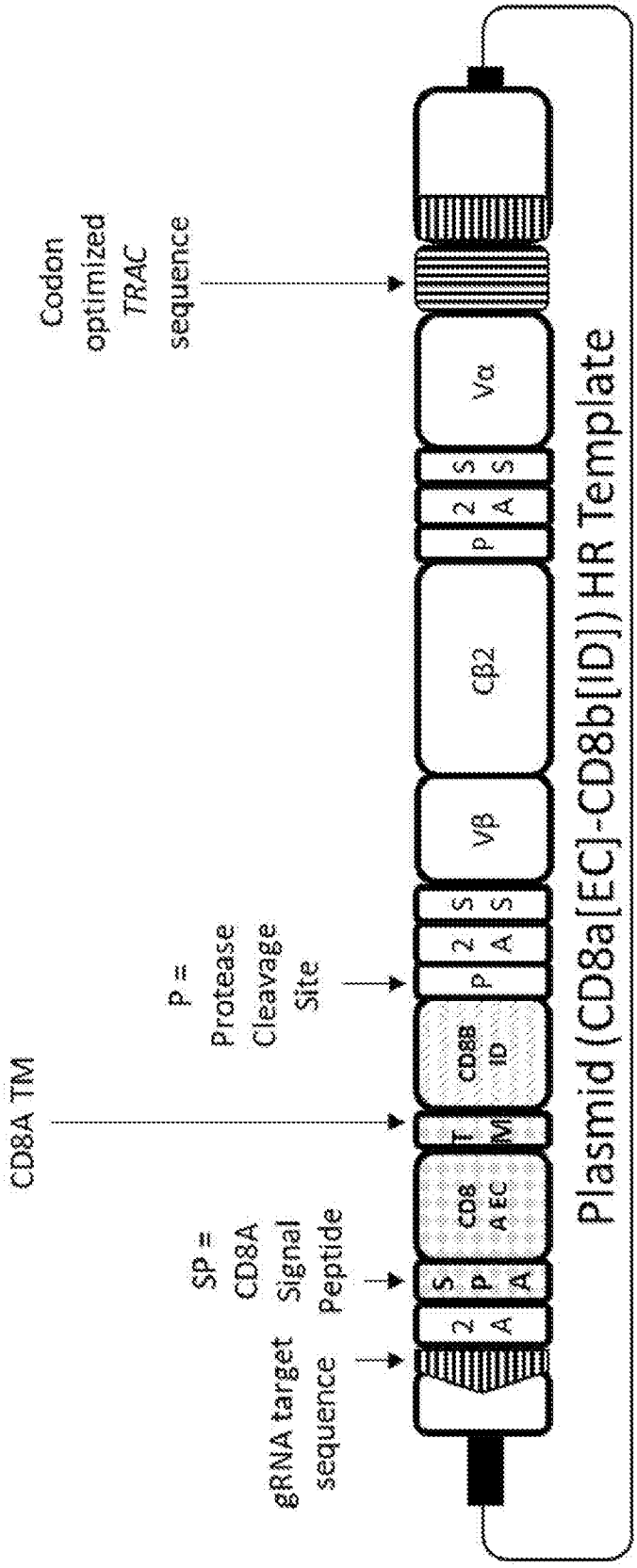

gRNA target sequence
SPA = CD8A Signal Peptide
CD8A TM
P = Protease Cleavage Site
SPB = CD8B Signal Peptide
CD8B TM
Codon optimized TRAC sequence
Vα
Cβ2
Vβ
Plasmid (CD8alpha-P2A-CD8beta) HR Template
CD8 Product 2

FIGURE 2C



CD8 Product 3

FIGURE 2D

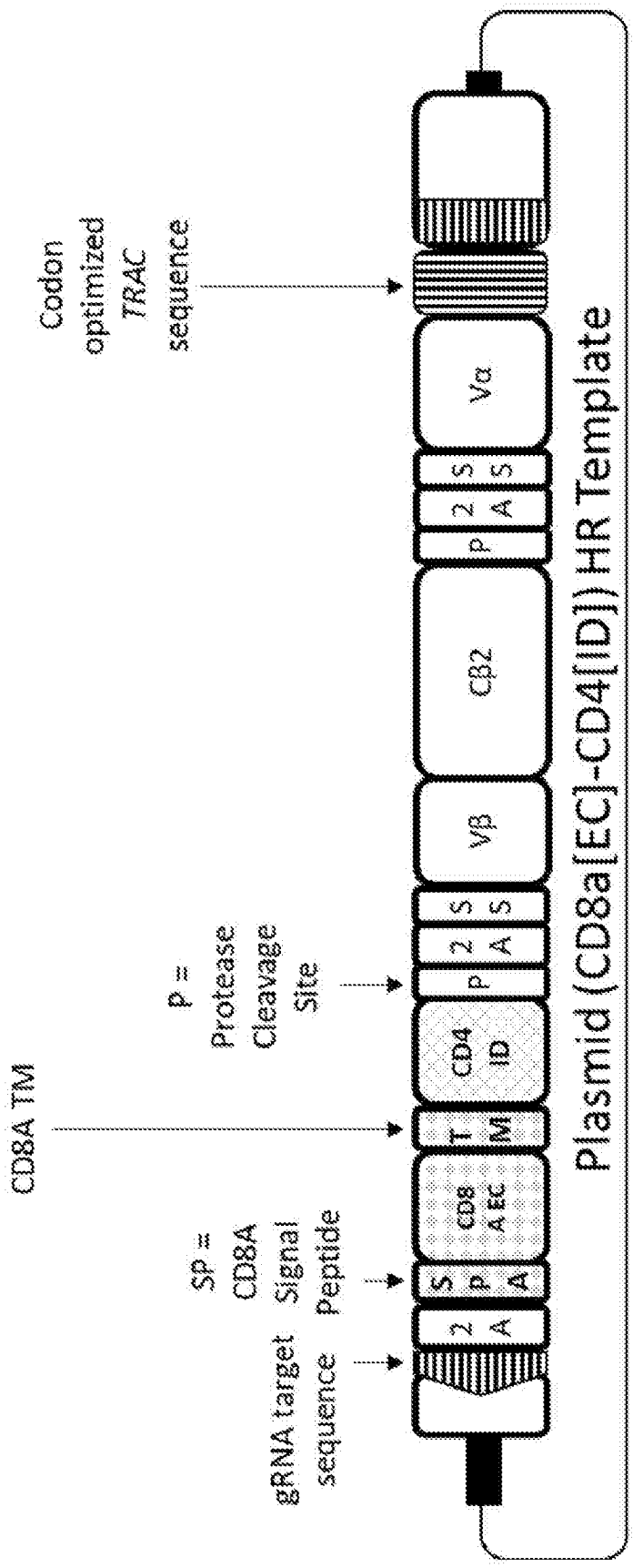

Codon optimized TRAC sequence
CD8A TM
P = Protease Cleavage Site
SP = CD8A Signal Peptide
gRNA target sequence
Vα
Cβ2
Vβ
CD4 ID
CD8 AEC
Plasmid (CD8a[EC]-CD4[ID]) HR Template
CD8 Product 4

FIGURE 3A

(CD8A_CD8 Product 1)

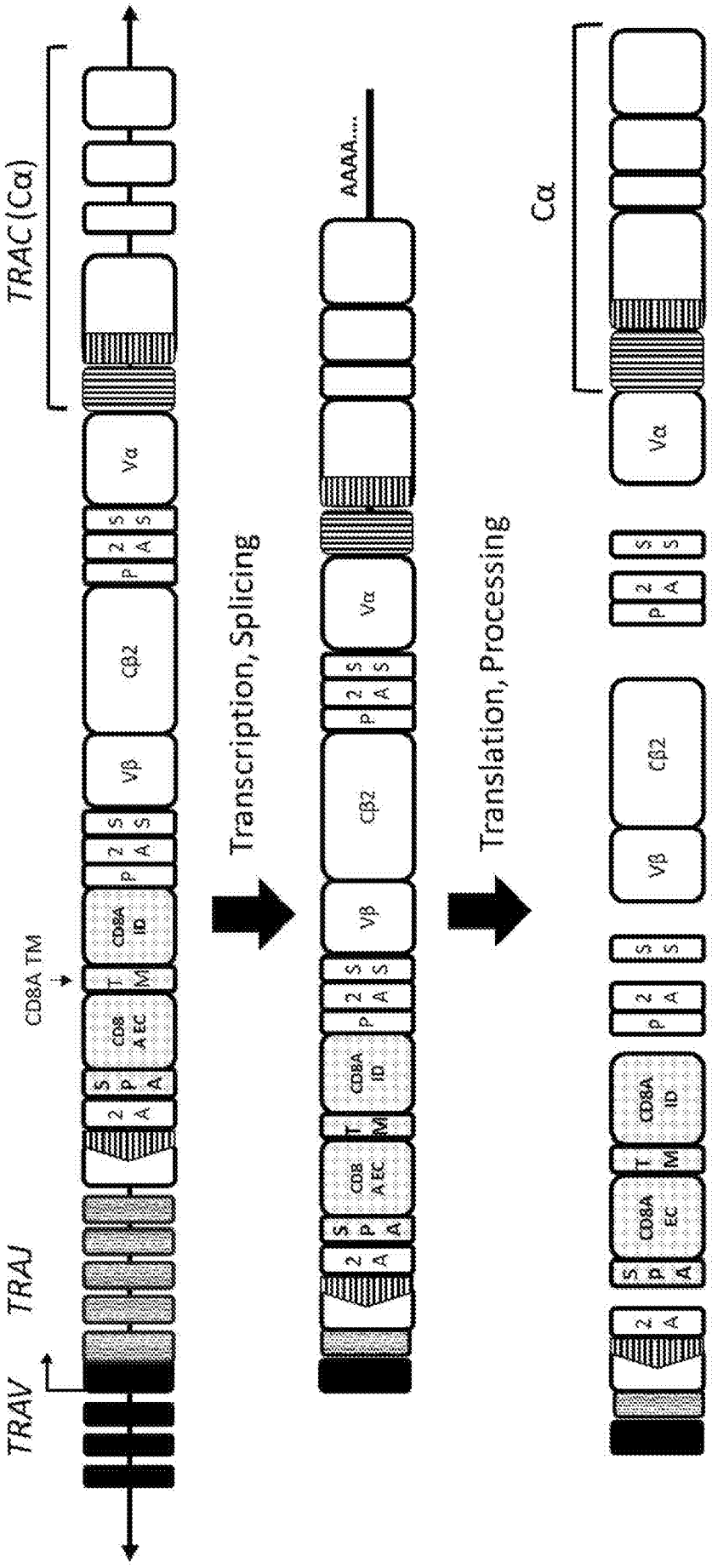


FIGURE 3B

(CD8alpha-P2A-CD8beta_CD8 Product 2)

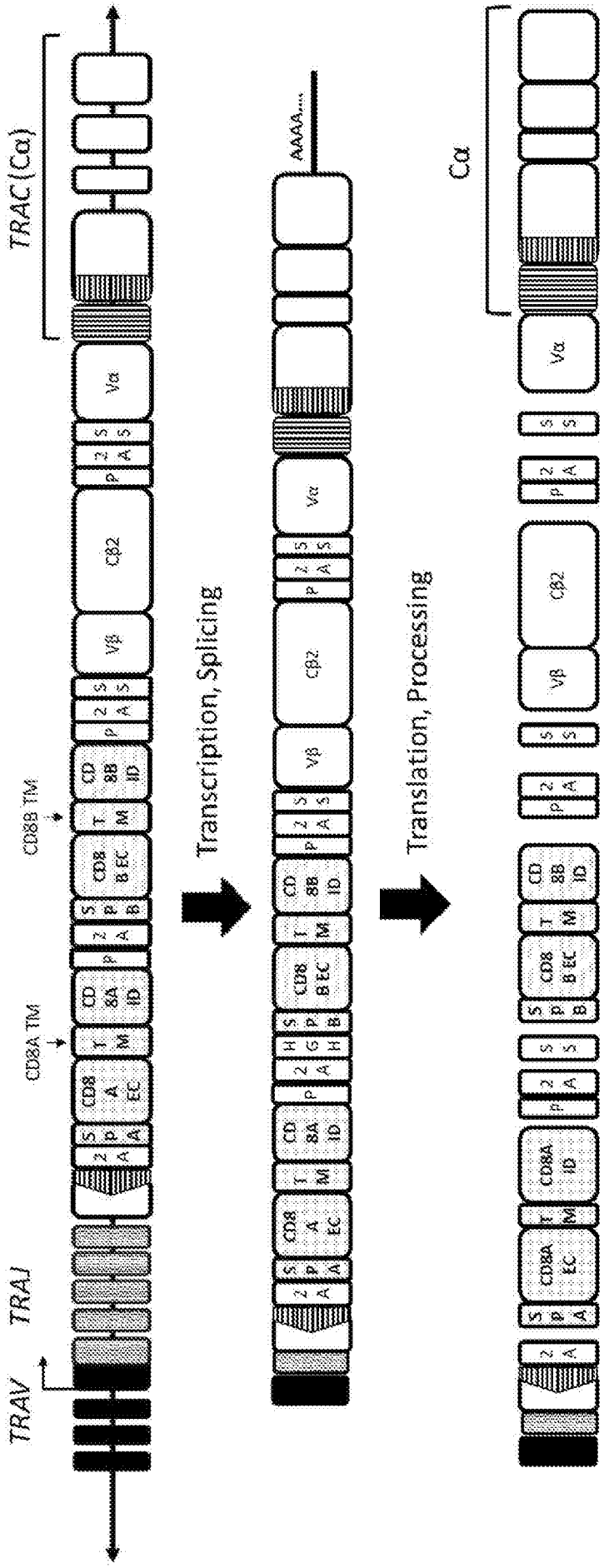


FIGURE 3C

(CD8A[EC]-CD8B[ID]_CD8 Product 3)

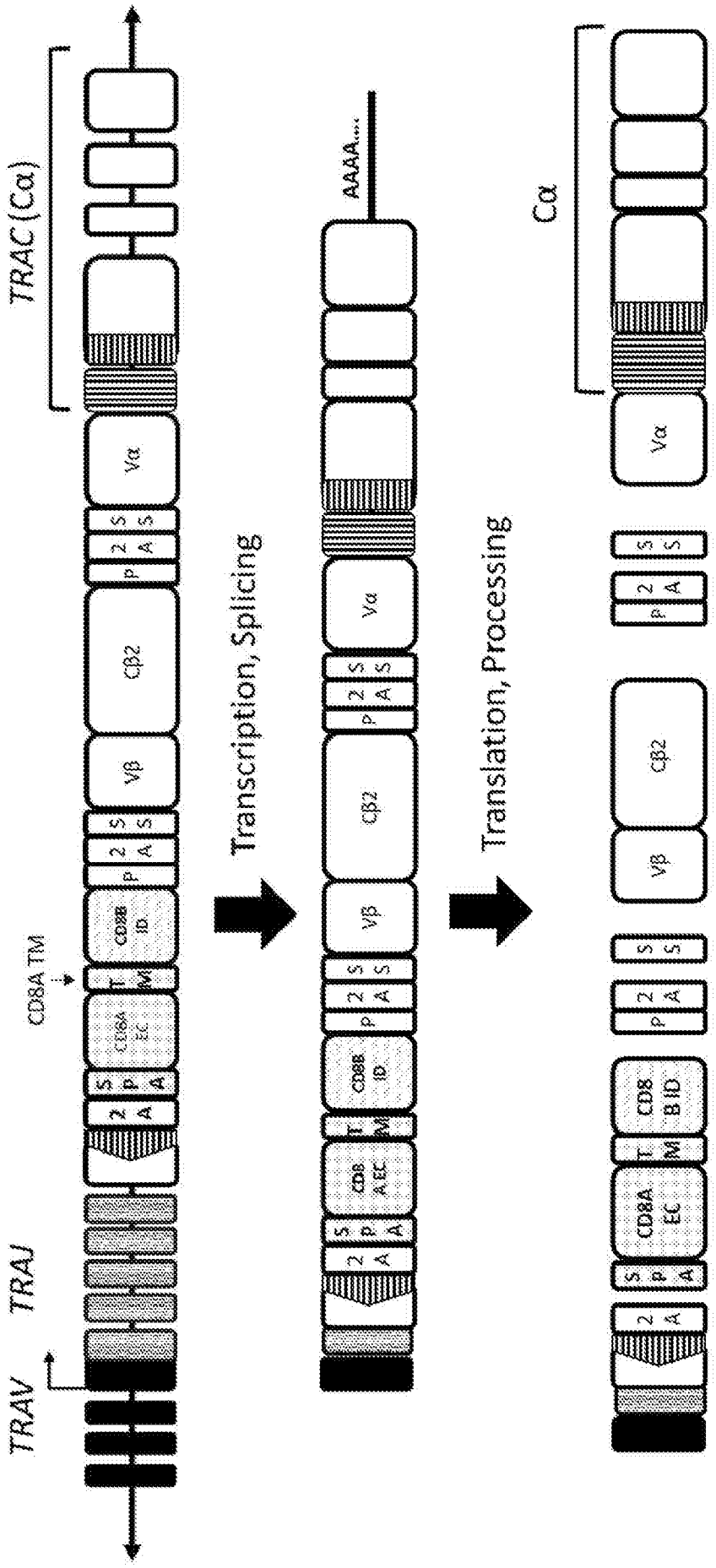


FIGURE 3D

(CD8a[EC]-CD4[ID]_CD8 Product 4)

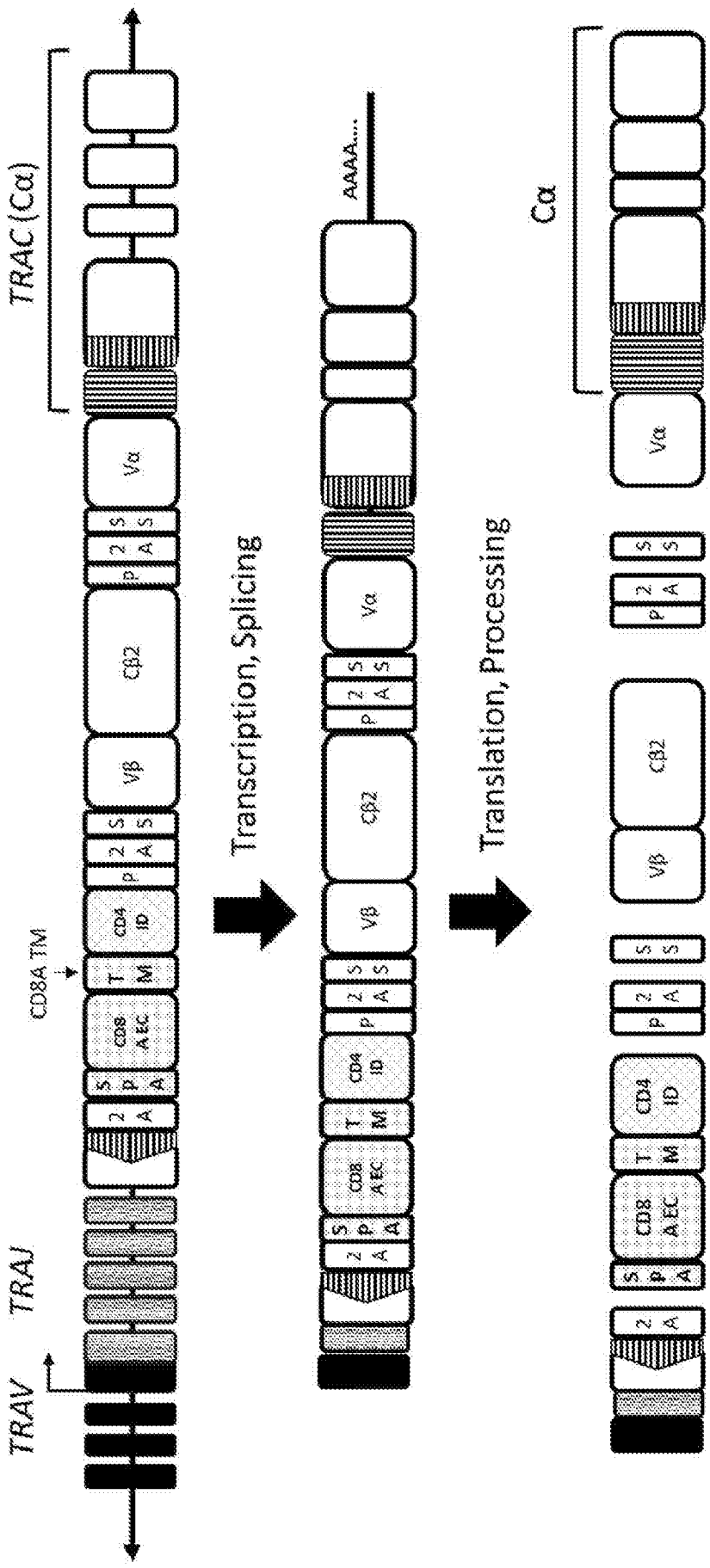


FIGURE 4A

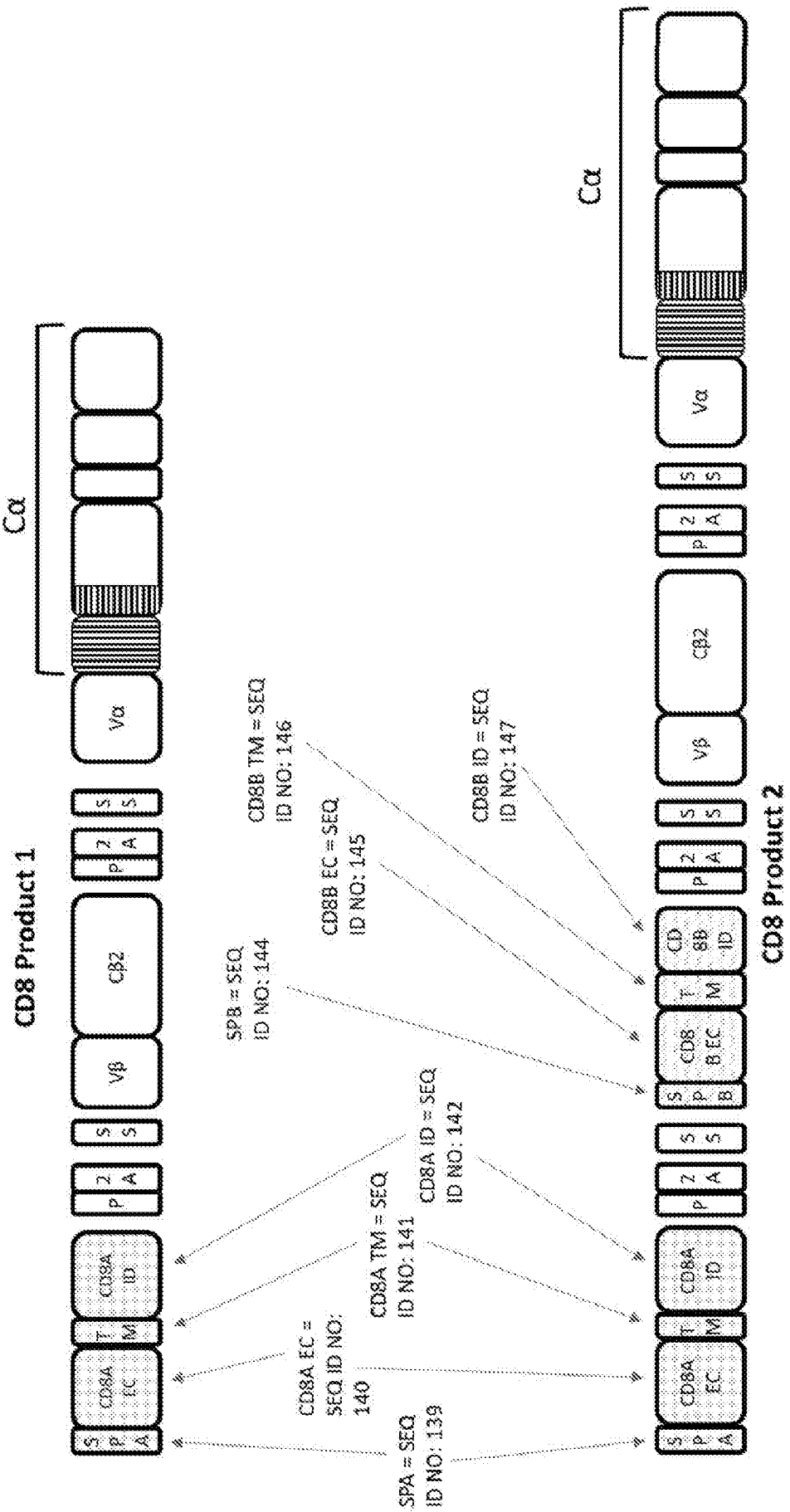

CD8 Product 1
Cα
Vα
Cβ2
Vβ
CD8A EC
CD8A TM
CD8A ID
SPA = SEQ ID NO: 139
CD8A EC = SEQ ID NO: 140
CD8A TM = SEQ ID NO: 141
CD8A ID = SEQ ID NO: 142
SPB = SEQ ID NO: 144
CD8B EC = SEQ ID NO: 145
CD8B TM = SEQ ID NO: 146
CD8B ID = SEQ ID NO: 147
CD8 Product 2
Cα
Vα
Cβ2
Vβ
CD8B EC
CD8B ID

FIGURE 4B

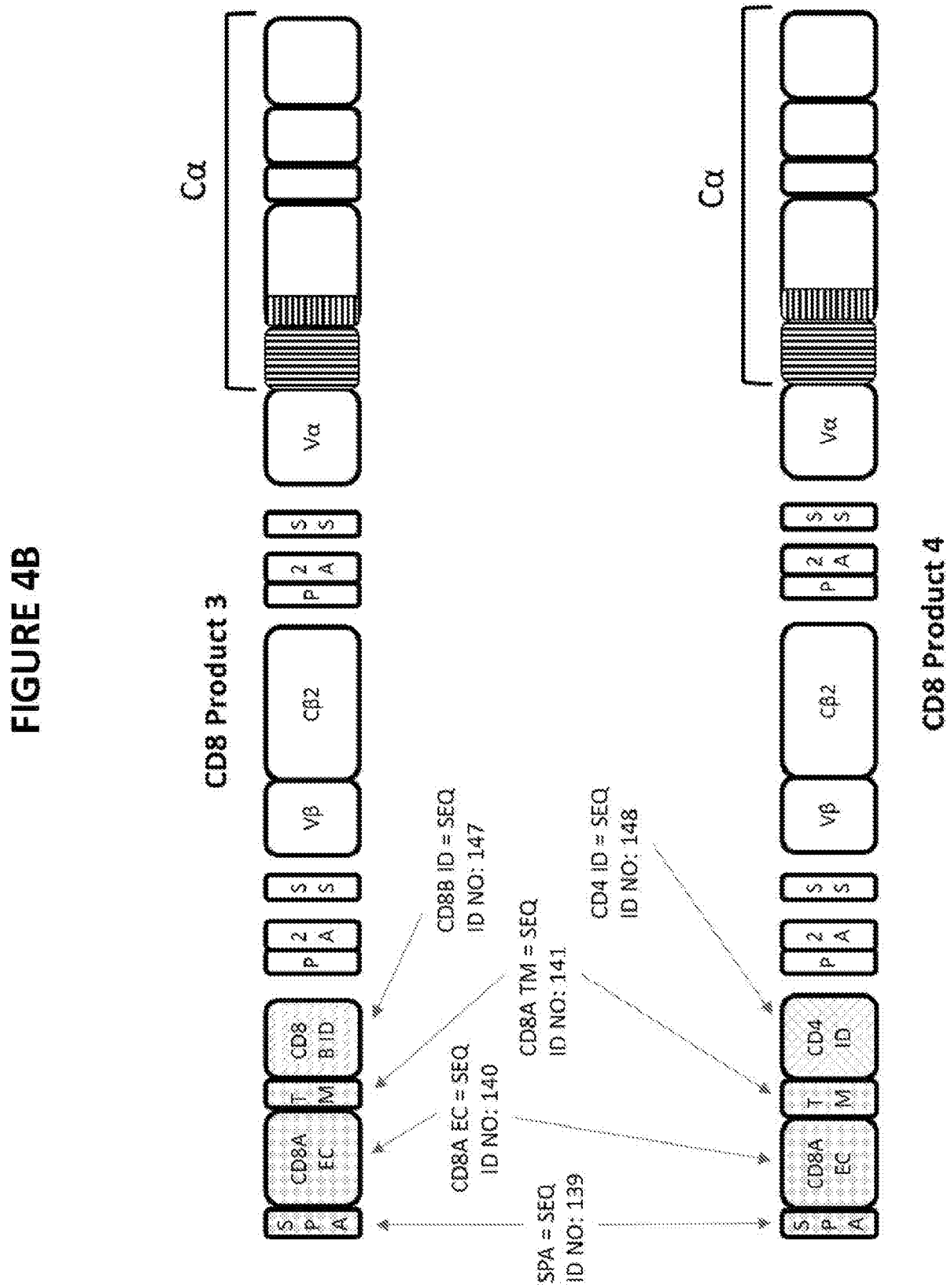

CD8 Product 3
Cα
Vα
Cβ2
Vβ
CD8 B ID
CD8A TM
CD8A EC
SPA
CD8B ID = SEQ ID NO: 147
CD8A TM = SEQ ID NO: 141
CD8A EC = SEQ ID NO: 140
SPA = SEQ ID NO: 139
CD4 ID = SEQ ID NO: 148
CD4 ID
CD8 Product 4

FIGURE 5

NeoTCR Product Nucleic Acid Sequence

ACATTAAAAACACAAAATCCTACGGAAATACTGAAGAATGAGTCTCAGCACTAAGGAAAAGC
CTCCAGCAGCTCCTGCTTTCTGAGGGTGAAGGATAGACGCTGTGGCTCTGCATGACTCACTA
GCACTCTATCACGGCCATATTCTGGCAGGGTCAGTGGCTCCAACTAACATTTGTTTGGTACTT
TACAGTTTATTAAATAGATGTTTATATGGAGAAGCTCTCATTTCTTTCTCAGAAGAGCCTGGCT
AGGAAGGTGGATGAGGCACCATATTCATTTTGCAGGTGAAATTCCTGAGATGTAAGGAGCT
GCTGTGACTTGCTCAAGGCCTTATATCGAGTAAACGGTAGTGCTGGGGCTTAGACGCAGGTG
TTCTGATTTATAGTTCAAAACCTCTATCAATGAGAGAGCAATCTCCTGGTAATGTGATAGATTT
CCCAACTTAATGCCAACATACCATAAACCTCCCATTCTGCTAATGCCCAGCCTAAGTTGGGGA
GACCACTCCAGATTCCAAGATGTACAGTTTGCTTTGCTGGGCCTTTTTCCCATGCCTGCCTTTA
CTCTGCCAGAGTTATATTGCTGGGGTTTTGAAGAAGATCCTATTAAATAAAAGAATAAGCAGT
ATTATTAAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACG
TTCACTGAAATCATGGCCTCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTC
CATCACGAGCAGCTGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTGCC
AGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGACTCCAGCCTGGGTTGGGGCA
AAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAG

Left HR Arm (SEQ ID NO:1)

ATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTG
ACAAGTCTGTCTGCCTATTC

TRAC CDS (SEQ ID NO:2)

GAATTCGGCTCCGGA

GSG Linker 1 (SEQ ID NO:3)

GCCACTAACTTCTCCCTGTTGAAACAGGCTGGCGATGTTGAAGAAAACCCCGG
TCCT

P2A (SEQ ID NO:4)

FIGURE 5 Cont.

ATGGCCACCGGCTCTAGAACAAGCCTGCTGCTCGCTTTTGGCCTGCTCTGCCTCCCATGGCTC
CAAGAAGGATCTGCT

HGH/SS/2 (SEQ ID NO:5)

[[TRB_VDJ Sequence, specific for the NeoTCR]]

Insertion site for neoTCR TRB_VDJ sequence

CTGAAAAACGTGTTCCCTCCAAAAGTGGCCGTGTTCGAGCCTTCTGAGGCCGAGATCAGCC
ACACACAGAAAGCCACACTCGTGTGTCTGGCTACCGGCTTCTACCCCGATCACGTGGAACTG
TCTTGGTGGGTCAACGGCAAAGAGGTGCACAGCGGCGTCAGCACAGATCCCCAGCCTCTGA
AAGAACAGCCCGCTCTGAACGACAGCCGCTACTGCCTGTCTAGCAGACTGAGAGTGTCCGC
CACCTTCTGGCAGAACCCCAGAAACCACTTCAGATGCCAGGTCCAGTTCTACGGCCTGAGCG
AGAACGATGAGTGGACCCAGGACAGAGCCAAGCCTGTGACACAGATCGTGTCTGCCGAAG
CCTGGGGCAGAGCCGATTGTGGCTTTACCAGCGAGTCATACCAGCAGGGCGTGCTGTCTGC
CACCATCCTGTATGAGATCCTGCTCGGCAAGGCCACACTGTACGCTGTGCTGGTGTCTGCTCT
GGTGCTGATGGCTATGGTCTCCCGGGAGCGCATCCCCGAGGCC

TCR-beta/constant (SEQ ID NO:7)

CGGGCCAAGCGG

Furin Cleavage Site (SEQ ID NO: 8)

GGCAGCGGC

GSG Linker (SEQ ID NO:9)

GCCACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACGTGGAGGAGAACCCCGGCCCT

P2A (SEQ ID NO:10)

ATGGCCACAGGCAGCAGAACATCTCTGCTGCTGGCCTTCGGACTGCTGTGTCTGCCTTGGCT
GCAAGAGGGTTCCGCC

HGH SS (SEQ ID NO:11)

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FIGURE 5 Cont.

[[TRA_VDJ Sequence, specific for the NeoTCR]]

Insertion site for neoTCR TRA_VDJ sequence

ATATTCAGAACCCCGATCCTGCTGTGTATCAGCTGCGCGACAGCAAGAGCAGCGACAAGAGC
GTGTGTTTGTTC

TCR-alpha/constant (SEQ ID NO:13)

ACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGAC
AAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCA
ACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCT
TCCCCAGCCCAGG

TRAC CDS/right HR arm(SEQ ID NO: 14)

TAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAATGGCCAGGTTCTGCC
CAGAGCTCTGGTCAATGATGTCTAAAACTCCTCTGATTGGTGGTCTCGGCCTTATCCATTGCC
ACCAAAACCCTCTTTTTACTAAGAAACAGTGAGCCTTGTTCTGGCAGTCCAGAGAATGACAC
GGGAAAAAAGCAGATGAAGAGAAGGTGGCAGGAGAGGGCACGTGGCCCAGCCTCAGTCT
CTCCAACTGAGTTCCTGCCTGCCTGCCTTTGCTCAGACTGTTTGCCCCTTACTGCTCTTCTAGG
CCTCATTCTAAGCCCCTTCTCCAAGTTGCCTCTCCTTATTTCTCCCTGTCTGCCAAAAAATCTTT
CCCAGCTCACTAAGTCAGTCTCACGCAGTCACTCATTAACCCACCAATCACTGATTGTGCCGG
CACATGAATGCACCAGGTGTTGAAGTGGAGGAATTAAAAAGTCAGATGAGGGGTGTGCCCA
GAGGAAGCACCATTCTAGTTGGGGGAGCCCATCTGTCAGCTGGGAAAAGTCCAAATAACTT
CAGATTGGAATGTGTTTTAACTCAGGGTTGAGAAAACAGCTACCTTCAGGACAAAAGTCAG
GGAAGGGCTCTCTGAAGAAATGCTACTTGAAGATACCAGCCCTACCAAGGGCAGGGAGAG
GACCCTATAGAGGCCTGGGACAGGAGCTCAATGAGAAAGGAGAAGAGCAGCAGGCATGA
GTTGAATGAAGGAGGCAGGGCCGGGTCACAGGGCCTTCTAGGCCATGAGAGGGTAGACAG

Right HR arm (SEQ ID NO:15)

GCTAGC

(SEQ ID NO:16)

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FIGURE 5 Cont.

pBR322_origin (SEQ ID NO:17)

CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAG
CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCT
TCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT
CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGG
TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTG
GTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCC
TAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTT
CGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCT

TTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCA
TATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGAT
GGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTC
CCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGA
GAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTC
ATCAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCCAGACGAAA
TACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAAC
ACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTG
TTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTG
ATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCA
TTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAAG
CGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAG
CATCCATGTTGGAATTTAATCGCGGCCTCGACGTTTCCCGTTGAATATGGCTCAT

AACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATC
TTGTGCAATGTAACATCAGAGATTTTGAGACAC

Kanamycin resistant gene (KanR2) (SEQ ID NO:18)

Kanamycin Promoter (SEQ ID NO:19)

FIGURE 6

CD8 Product 1 Product Nucleic Acid Sequence

ACATTAAAAACACAAAATCCTACGGAAATACTGAAGAATGAGTCTCAGCACTAAGGAAAAGC
CTCCAGCAGCTCCTGCTTTCTGAGGGTGAAGGATAGACGCTGTGGCTCTGCATGACTCACTA
GCACTCTATCACGGCCATATTCTGGCAGGGTCAGTGGCTCCAACTAACATTTGTTTGGTACTT
TACAGTTTATTAAATAGATGTTTATATGGAGAAGCTCTCATTTCTTTCTCAGAAGAGCCTGGCT
AGGAAGGTGGATGAGGCACCATATTCATTTTGCAGGTGAAATTCCTGAGATGTAAGGAGCT
GCTGTGACTTGCTCAAGGCCTTATATCGAGTAAACGGTAGTGCTGGGGCTTAGACGCAGGTG
TTCTGATTTATAGTTCAAAACCTCTATCAATGAGAGAGCAATCTCCTGGTAATGTGATAGATTT
CCCAACTTAATGCCAACATACCATAAACCTCCCATTCTGCTAATGCCCAGCCTAAGTTGGGGA
GACCACTCCAGATTCCAAGATGTACAGTTTGCTTTGCTGGGCCTTTTTCCCATGCCTGCCTTTA
CTCTGCCAGAGTTATATTGCTGGGGTTTTGAAGAAGATCCTATTAAATAAAAGAATAAGCAGT
ATTATTAAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACG
TTCACTGAAATCATGGCCTCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTC
CATCACGAGCAGCTGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTGCC
AGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGACTCCAGCCTGGGTTGGGGCA
AAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAG

Left HR Arm (SEQ ID NO:22)

ATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTG
TCTGCCTATTC

TRAC CDS (SEQ ID NO:23)

GAATTCGGCTCCGGA

GSG Linker 1 (SEQ ID NO:24)

GCCACTAACTTCAGCCTGTTGAAGCAGGCCGGCGACGTTGAGGAAAACCCCGGTCCT

P2A (SEQ ID NO:25)

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FIGURE 6 Cont.

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGGCCG

CD8A Signal peptide (SEQ ID NO:26)

AGCCAGTTCCGGGTGTCGCCGCTGGATCGGACCTGGAACCTGGGCGAGACAGTGGAGCTG
AAGTGCCAGGTGCTGCTGTCCAACCCGACGTCGGGCTGCTCGTGGCTCTTCCAGCCGCGCG
GCGCCGCCGCCAGTCCCACCTTCCTCCTATACCTCTCCCAAAACAAGCCCAAGGCGGCCGAG
GGGCTGGACACCCAGCGGTTCTCGGGCAAGAGGTTGGGGGACACCTTCGTCCTCACCCTG
AGCGACTTCCGCCGAGAGAACGAGGGCTACTATTTCTGCTCGGCCCTGAGCAACTCCATCAT
GTACTTCAGCCACTTCGTGCCGGTCTTCCTGCCAGCGAAGCCCACCACGACGCCAGCGCCGC
GACCACCAACACCGGCGCCCACCATCGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGTG
CCGGCCAGCGGCGGGGGGCGCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGAT

CD8A Extracellular domain (SEQ ID NO:27)

CD8A transmembrane domain (SEQ ID NO:28)

ATCTACATCTGGGCGCCCTTGGCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTTATCACC

CTTTACTGCAACCACAGGAACCGAAGACGTGTTTGCAAATGTCCCCGGCCTGTGGTCAAATC
GGGAGACAAGCCCAGCCTTTCGGCGAGATACGTC

CD8A Intracellular domain (SEQ ID NO:29)

Furin cleavage site (SEQ ID NO:30)

AGGGCTAAACGG

GSG Linker (SEQ ID NO:31)

GAATTCGGCTCCGGA

P2A (SEQ ID NO:32)

GCCACTAACTTCTCCCTGTTGAAACAGGCTGGCGATGTTGAAGAAAACCCCGGTCCT

HGH/SS/2 (SEQ ID NO:33)

ATGGCCACCGGCTCTAGAACAAGCCTGCTCGCTTTTGGCCTGCTCTGCCTCCCATGGCTC
CAAGAAGGATCTGCT

FIGURE 6 Cont.

[[TRB_VDJ Sequence, specific for the NeoTCR]] **Insertion site for neoTCR TRB_VDJ sequence**

CTGAAAAACGTGTTCCCTCCAAAAGTGGCCGTGTTCGAGCCTTCTGAGGCCGAGATCAGCC
ACACACAGAAAGCCACACTCGTGTGTCTGGCTACCGGCTTCTACCCCGATCACGTGGAACTG
TCTTGGTGGGTCAACGGCAAAGAGGTGCACAGCGGCGTCAGCACAGATCCCCAGCCTCTGA
AAGAACAGCCCGCTCTGAACGACAGCCGCTACTGCCTGTCTAGCAGACTGAGAGTGTCCGC
CACCTTCTGGCAGAACCCCAGAAACCACTTCAGATGCCAGGTCCAGTTCTACGGCCTGAGCG
AGAACGATGAGTGGACCCAGGACAGAGCCAAGCCTGTGACACAGATCGTGTCTGCCGAAG
CCTGGGGCAGAGCCGATTGTGGCTTTACCAGCGAGTCATACCAGCAGGGCGTGCTGTCTGC
CACCATCCTGTATGAGATCCTGCTCGGCAAGGCCACACTGTACGCTGTGCTGGTGTCTGCTCT
GGTGCTGATGGCTATGGTCTCCCGGGAGCGCATCCCCGAGGCC

TCR-beta/constant (SEQ ID NO:35)

CGGGCCAAGCGG **Furin cleavage site (SEQ ID NO:36)**

GGCAGCGGC **GSG linker (SEQ ID NO:37)**

GCCACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACGTGGAGGAGAACCCCGGCCCT **P2A (SEQ ID NO:38)**

ATGGCCACAGGCAGCAGAACATCTCTGCTGCTGGCCTTCGGACTGCTGTGTCTGCCTTGGCT GCAAGAGGGTTCCGCC **HGH/SS (SEQ ID NO:39)**

[[TRA_VDJ Sequence, specific for the NeoTCR]] **Insertion site for neoTCR TRA_VDJ sequence**

ATATTCAGAACCCCGATCCTGCTGTGTATCAGCTGCGCGACAGCAAGAGCAGCGACAAGAGC GTGTGTTTGTTC **TCR-alpha/constant (SEQ ID NO:41)**

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FIGURE 6 Cont.

TRAC CDS/right HR arm (SEQ ID NO:42)

ACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGAC
AAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCA
ACAAATCTGACTTTGCATGTGCAAACGCCTTCAACACAACAGCATTATTCCAGAAGACACCTTCT
TCCCCAGCCCAGG

Right HR arm (SEQ ID NO:43)

TAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAATGGCCAGGTTCTGCC
CAGAGCTCTGGTCAATGATGTCTAAAACTCCTCTGATTGGTGGTCTCGGCCTTATCCATTGCC
ACCAAAACCCTCTTTTTACTAAGAAACAGTGAGCCTTGTTCTGGCAGTCCAGAGAATGACAC
GGGAAAAAAGCAGATGAAGAAGGTGGCAGGAGAGGGCACGTGGCCCAGCCTCAGTCT
CTCCAACTGAGTTCCTGCCTGCCTTTGCTCAGACTGTTTGCCCCTTACTGCTCTTCTAGG
CCTCATTCTAAGCCCCTTCTCCAAGTTGCCTCTCCTTATTTCTCCCTGTCTGCCAAAAAATCTTT
CCCAGCTCACTAAGTCAGTCTCACGCAGTCACTCATTAACCCACCAATCACTGATTGTGCCGG
CACATGAATGCACCAGGTGTTGAAGTGGAGGAATTAAAAAGTCAGATGAGGGGTGTGCCCA
GAGGAAGCACCATTCTAGTTGGGGGAGCCCATCTGTCAGCTGGGAAAAGTCCAAATAACTT
CAGATTGGAATGTGTTTTAACTCAGGGTTGAGAAAACAGCTACCTTCAGGACAAAAGTCAG
GGAAGGGCTCTCTGAAGAAATGCTACTTGAAGATACCAGCCCTACCAAGGGCAGGGAGAG
GACCCTATAGAGGCCTGGGACAGGAGCTCAATGAGAAAGGAGAAGAGCAGCAGGCATGA
GTTGAATGAAGGAGGCAGGGCCGGGTCACAGGGCCTTCTAGGCCATGAGAGGGTAGACAG

(SEQ ID NO:44)

GCTAGC

FIGURE 6 Cont.

pBR322_origin (SEQ ID NO:45)

CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAG
CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCT
TCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT
CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGG
TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTG
GTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCC
TAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTT
CGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCT

TTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCA
TATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGAT
GGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTC
CCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGA
GAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTC
ATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCCAGACGAAA
TACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAAC
ACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTG
TTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTG
ATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCA
TTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAAG
CGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAG
CATCCATGTTGGAATTTAATCGCGGCCTCGACGTTTCCCGTTGAATATGGCTCAT

AACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATC
TTGTGCAATGTAACATCAGAGATTTTGAGACAC

Kanamycin resistance (KanR2) (SEQ ID NO:46)

Kanamycin promoter (SEQ ID NO:47)

FIGURE 7

CD8 Product 2 Product Nucleic Acid Sequence

ACATTAAAAACACAAAATCCTACGGAAATACTGAAGAATGAGTCTCAGCACTAAGGAAAAGC
CTCCAGCAGCTCCTGCTTTCTGAGGGTGAAGGATAGACGCTGTGGCTCTGCATGACTCACTA
GCACTCTATCACGGCCATATTCTGGCAGGGTCAGTGGCTCCAACTAACATTTGTTTGGTACTT
TACAGTTTATTAAATAGATGTTTATATGGAGAAGCTCTCATTTCTTTCTCAGAAGAGCCTGGCT
AGGAAGGTGGATGAGGCACCATATTCATTTTGCAGGTGAAATTCCTGAGATGTAAGGAGCT
GCTGTGACTTGCTCAAGGCCTTATATCGAGTAAACGGTAGTGCTGGGGCTTAGACGCAGGTG
TTCTGATTTATAGTTCAAAACCTCTATCAATGAGAGAGCAATCTCCTGGTAATGTGATAGATTT
CCCAACTTAATGCCAACATACCATAAACCTCCCATTCTGCTAATGCCCAGCCTAAGTTGGGGA
GACCACTCCAGATTCCAAGATGTACAGTTTGCTTTGCTGGGCCTTTTTCCCATGCCTGCCTTTA
CTCTGCCAGAGTTATATTGCTGGGGTTTTGAAGAAGATCCTATTAAATAAAAGAATAAGCAGT
ATTATTAAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACG
TTCACTGAAATCATGGCCTCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTC
CATCACGAGCAGCTGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTGCC
AGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGACTCCAGCCTGGGTTGGGGCA
AAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAG

Left HR Arm (SEQ ID NO:49)

ATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTG
TCTGCCTATTC

TRAC CDS (SEQ ID NO:50)

GAATTCGGCTCCGGA

GSG Linker 1 (SEQ ID NO:51)

GCCACTAACTTCAGCCTGTTGAAGCAGGCCGGCGACGTTGAGGAAAACCCCGGTCCT

P2A (SEQ ID NO:52)

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FIGURE 7 Cont.

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGGCCG

CD8A Signal peptide (SEQ ID NO:53)

AGCCAGTTCCGGGGTGTCGCCGCTGGATCGGACCTGGAACCTGGGCGAGACAGTGGAGCTG
AAGTGCCAGGTGCTGCTGTCCAACCCGACGTCGGGCTGCTCGTGGCTCTTCCAGCCGCGCG
GCGCCGCCGCCAGTCCCACCTTCCTCCTATACCTCTCCCAAAACAAGCCCAAGGCGGCCGAG
GGGCTGGACACCCAGCGGTTCTCGGGCAAGAGGTTGGGGGACACCTTCGTCCTCACCCTG
AGCGACTTCCGCCGAGAGAACGAGGGCTACTATTTCTGCTCGGCCCTGAGCAACTCCATCAT
GTACTTCAGCCACTTCGTGCCGGTCTTCCTGCCAGCGAAGCCCACCACGACGCCAGCGCCGC
GACCACCAACACCGGCGCCCACCATCGCGTCGCAGCCCCCTGTCCCTGCGCCCAGAGGCGTG
CCGGCCAGCGGCGGGGGGCGCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGAT

CD8A Extracellular domain (SEQ ID NO:54)

CD8A transmembrane domain (SEQ ID NO:55)

ATCTACATCTGGGCGCCCTTGGCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTTATCACC

CTTTACTGCAACCACAGGAACCGAAGACGTGTTTGCAAATGTCCCCGGCCTGTGGTCAAATC
GGGAGACAAGCCCAGCCTTTCGGCGAGATACGTC

CD8A Intracellular domain (SEQ ID NO:56)

AGAGCAAAGCGG

Furin cleavage site (SEQ ID NO:57)

GGCTCCGGA

GSG Linker (SEQ ID NO:58)

GCTACCAATTTTAGCCTCCTGAAGCAGGCTGGCGATGTTGAGGAAAACCCTGGTCCC

P2A (SEQ ID NO:59)

ATGCGGCCGCGGCTGTGGCTCCTCTTGGCCGCGCAGCTGACAGTTCTCCATGGCAACTCAGT
C

CD8B Signal Peptide (SEQ ID NO:60)

FIGURE 7 Cont.

CTCCAGCAGACCCCTGCATACATAAAGGTGCAAACCAACAAGATGGTGATGCTGTCCTGCGA
GGCTAAAATCTCCCTCAGTAACATGCGCATCTACTGGCTGAGACAGCGCCAGGCACCGAGCA
GTGACAGTCACCACGAGTTCCTGGCCCTCTGGGATTCCGCAAAAGGGACTATCCACGGTGA
AGAGGTGGAACAGGAGAAGATAGCTGTGTTTCGGGATGCAAGCCGGTTCATTCTCAATCTC
ACAAGCGTGAAGCCGGAAGACAGTGGCATCTACTTCTGCATGATCGTCGGGAGCCCCGAGC
TGACCTTCGGGAAGGGAACTCAGCTGAGTGTGGTTGATTTCCTTCCCACCACTGCCCAGCCC
ACCAAGAAGTCCACCCTCAAGAAGAGAGTGTGCCGGTTACCCAGGCCAGAGACCCAGAAG
GGCCCACTTTGTAGCCCC

CD8B Extracellular domain (SEQ ID NO:61)

ATCACCCTTGGCCTGCTGGTGGCTGGCGTCCTGGTTCTGCTGGTTTCCCTGGGAGTGGCCAT
C

CD8B Transmembrane domain (SEQ ID NO:62)

CACCTGTGCTGCCGGCGGAGGAGAGCCCGGCTTCGTTTCATGAAACAATTTTACAAA

CD8B Intracellular domain (SEQ ID NO:63)

AGGGCTAAACGG

Furin cleavage site (SEQ ID NO:64)

GAATTCGGCTCCGGA

GSG Linker (SEQ ID NO:65)

GCCACTAACTTCTCCCTGTTGAAACAGGCTGGCGATGTTGAAGAAAACCCCGGTCCT

P2A (SEQ ID NO:66)

ATGGCCACCGGCTCTAGAACAAGCCTGCTGCTCGCTTTTGGCCTGCTCTGCCTCCCATGGCTC
CAAGAAGGATCTGCT

HGH/SS/2 (SEQ ID NO:67)

FIGURE 7 Cont.

[[TRB_VDJ Sequence, specific for the NeoTCR]]

Insertion site for neoTCR TRB_VDJ sequence

CTGAAAAACGTGTTCCCTCCAAAAGTGGCCGTGTTCGAGCCTTCTGAGGCCGAGATCAGCC
ACACACAGAAAGCCACACTCGTGTGTCTGGCTACCGGCTTCTACCCCGATCACGTGGAACTG
TCTTGGTGGGTCAACGGCAAAGAGGTGCACAGCGGCGTCAGCACAGATCCCCAGCCTCTGA
AAGAACAGCCCGCTCTGAACGACAGCCGCTACTGCCTGTCTAGCAGACTGAGAGTGTCCGC
CACCTTCTGGCAGAACCCCAGAAACCACTTCAGATGCCAGGTCCAGTTCTACGGCCTGAGCG
AGAACGATGAGTGGACCCAGGACAGAGCCAAGCCTGTGACACAGATCGTGTCTGCCGAAG
CCTGGGGCAGAGCCGATTGTGGCTTTACCAGCGAGTCATACCAGCAGGGCGTGCTGTCTGC
CACCATCCTGTATGAGATCCTGCTCGGCAAGGCCACACTGTACGCTGTGCTGGTGTCTGCTCT
GGTGCTGATGGCTATGGTCTCCCGGGAGCGCATCCCCGAGGCC

TCR-beta/constant (SEQ ID NO:69)

CGGGCCAAGCGG

Furin cleavage site (SEQ ID NO:70)

GGCAGCGGC

GSG Linker (SEQ ID NO:71)

GCCACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACGTGGAGGAGAACCCCGGCCCT

P2A (SEQ ID NO:72)

ATGGCCACAGGCAGCAGAACATCTCTGCTGCTGGCCTTCGGACTGCTGTGTCTGCCTTGGCT
GCAAGAGGGTTCCGCC

HGH/SS/2 (SEQ ID NO:73)

[[TRA_VDJ Sequence, specific for the NeoTCR]]

Insertion site for neoTCR TRA_VDJ sequence

ATATTCAGAACCCCGATCCTGCTGTGTATCAGCTGCGCGACAGCAAGAGCAGCGACAAGAGC
GTGTGTTTGTTC

TCR-alpha/constant (SEQ ID NO:75)

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FIGURE 7 Cont.

TRAC CDS/right HR arm (SEQ ID NO:76)

ACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGAC
AAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCA
ACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCT
TCCCCAGCCCAGG

TAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAATGGCCAGGTTCTGCC
CAGAGCTCTGGTCAATGATGTCTAAAACTCCTCTGATTGGTGGTCTCGGCCTTATCCATTGCC
ACCAAAACCCTCTTTTTACTAAGAAACAGTGAGCCTTGTTCTGGCAGTCCAGAGAATGACAC
GGGAAAAAAGCAGATGAAGAAGAAGGTGGCAGGAGAGGGCACGTGGCCCAGCCTCAGTCT
CTCCAACTGAGTTCCTGCCTGCCTGCCTTTGCTCAGACTGTTTGCCCCTTACTGCTCTTCTAGG
CCTCATTCTAAGCCCCTTCTCCAAGTTGCCTCTCCTTATTTCTCCCTGTCTGCCAAAAAATCTTT
CCCAGCTCACTAAGTCAGTCTCACGCAGTCACTCATTAACCCACCAATCACTGATTGTGCCGGG
CACATGAATGCACCAGGTGTTGAAGTGGAGGAATTAAAAAGTCAGATGAGGGGTGTGCCCA
GAGGAAGCACCATTCTAGTTGGGGGAGCCCATCTGTCAGCTGGGAAAAGTCCAAATAACTT
CAGATTGGAATGTGTTTTAACTCAGGGTTGAGAAAACAGCTACCTTCAGGACAAAAGTCAG
GGAAGGGCTCTCTGAAGAAATGCTACTTGAAGATACCAGCCCTACCAAGGGCAGGGAGAG
GACCCTATAGAGGCCTGGGACAGGAGCTCAATGAGAAAGGAGAAGAGCAGCAGGCATGA
GTTGAATGAAGGAGGCAGGGCCGGGTCACAGGGCCTTCTAGGCCATGAGAGGGTAGACAG

GCTAGC

Right HR arm (SEQ ID NO:77)

(SEQ ID NO:78)

FIGURE 7 Cont.

pBR322_origin (SEQ ID NO:79)

CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAG
CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCT
TCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT
CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGG
TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTG
GTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCC
TAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTT
CGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCT

TTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCA
TATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGAT
GGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTC
CCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGA
GAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTC
ATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCCAGACGAAA
TACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAAC
ACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTG
TTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTG
ATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCA
TTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAAG
CGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAG
CATCCATGTTGGAATTTAATCGCGGCCTCGACGTTTCCCGTTGAATATGGCTCAT

AACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATC
TTGTGCAATGTAACATCAGAGATTTTGAGACAC

Kanamycin resistance (KanR2) (SEQ ID NO:80)

Kanamycin promoter (SEQ ID NO:81)

FIGURE 8

CD8 Product 3 Product Nucleic Acid Sequence

ACATTAAAAACACAAAATCCTACGGAAATACTGAAGAATGAGTCTCAGCACTAAGGAAAAGC
CTCCAGCAGCTCCTGCTTTCTGAGGGTGAAGGATAGACGCTGTGGCTCTGCATGACTCACTA
GCACTCTATCACGGCCATATTCTGGCAGGGTCAGTGGCTCCAACTAACATTTGTTTGGTACTT
TACAGTTTATTAAATAGATGTTTATATGGAGAAGCTCTCATTTCTTTCTCAGAAGAGCCTGGCT
AGGAAGGTGGATGAGGCACCATATTCATTTTGCAGGTGAAATTCCTGAGATGTAAGGAGCT
GCTGTGACTTGCTCAAGGCCTTATATCGAGTAAACGGTAGTGCTGGGGCTTAGACGCAGGTG
TTCTGATTTATAGTTCAAAACCTCTATCAATGAGAGAGCAATCTCCTGGTAATGTGATAGATTT
CCCAACTTAATGCCAACATACCATAAACCTCCCATTCTGCTAATGCCCAGCCTAAGTTGGGGA
GACCACTCCAGATTCCAAGATGTACAGTTTGCTTTGCTGGGCCTTTTTCCCATGCCTGCCTTTA
CTCTGCCAGAGTTATATTGCTGGGGTTTTGAAGAAGATCCTATTAAATAAAAGAATAAGCAGT
ATTATTAAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACG
TTCACTGAAATCATGGCCTCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTC
CATCACGAGCAGCTGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTGCC
AGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGACTCCAGCCTGGGTTGGGGCA
AAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAG

Left HR Arm (SEQ ID NO:83)

ATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTG
TCTGCCTATTC

TRAC CDS (SEQ ID NO:84)

GAATTCGGCTCCGGA

GSG Linker 1 (SEQ ID NO:85)

GCCACTAACTTCAGCCTGTTGAAGCAGGCCGGCGACGTTGAGGAAAACCCCGGTCCT

P2A (SEQ ID NO:86)

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FIGURE 8 Cont.

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGGCC
G

CD8A Signal peptide (SEQ ID NO:87)

AGCCAGTTCCGGGTGTCGCCGCTGGATCGGACCTGGAACCTGGGCGAGACAGTGGAGCTG
AAGTGCCAGGTGCTGCTGTCCAACCCGACGTCGGGCTGCTCGTGGCTCTTCCAGCCGCGCG
GCGCCGCCGCCAGTCCCACCTTCCTCCTATACCTCTCCCAAAACAAGCCCAAGGCGGCCGAG
GGGCTGGACACCCAGCGGTTCTCGGGCAAGAGGTTGGGGGACACCTTCGTCCTCACCCTG
AGCGACTTCCGCCGAGAGAACGAGGGCTACTATTTCTGCTCGGCCCTGAGCAACTCCATCAT
GTACTTCAGCCACTTCGTGCCGGTCTTCCTGCCAGCGAAGCCCACCACGACGCCAGCGCCGC
GACCACCAACACCGGCGCCCACCATCGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGTG
CCGGCCAGCGGCGGGGGCGCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGAT

CD8A Extracellular domain (SEQ ID NO:88)

CD8A transmembrane domain (SEQ ID NO:89)

ATCTACATCTGGGCGCCCTTGGCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTTATCACC

CD8B Intracellular domain (SEQ ID NO:90)

CACCTGTGCTGCCGGCGGAGGAGAGCCCGGCTTCGTTTCATGAAACAATTTTACAAA

Furin cleavage site (SEQ ID NO:91)

AGGGCTAAACGG

GSG Linker (SEQ ID NO:92)

GAATTCGGCTCCGGA

P2A (SEQ ID NO:93)

GCCACTAACTTCTCCCTGTTGAAACAGGCTGGCGATGTTGAAGAAAACCCCGGTCCT

HGH/SS/2 (SEQ ID NO:94)

ATGGCCACCGGCTCTAGAACAAGCCTGCTCGCTTTTTGGCCTGCTCTGCCTCCCATGGCTC
CAAGAAGGATCTGCT

FIGURE 8 Cont.

[[TRB_VDJ Sequence, specific for the NeoTCR]]

Insertion site for neoTCR TRB_VDJ sequence

CTGAAAAACGTGTTCCCTCCAAAAGTGGCCGTGTTCGAGCCTTCTGAGGCCGAGATCAGCC
ACACACAGAAAGCCACACTCGTGTGTCTGGCTACCGGCTTCTACCCCGATCACGTGGAACTG
TCTTGGTGGGTCAACGGCAAAGAGGTGCACAGCGGCGTCAGCACAGATCCCCAGCCTCTGA
AAGAACAGCCCGCTCTGAACGACAGCCGCTACTGCCTGTCTAGCAGACTGAGAGTGTCCGC
CACCTTCTGGCAGAACCCCAGAAACCACTTCAGATGCCAGGTCCAGTTCTACGGCCTGAGCG
AGAACGATGAGTGGACCCAGGACAGAGCCAAGCCTGTGACACAGATCGTGTCTGCCGAAG
CCTGGGGCAGAGCCGATTGTGGCTTTACCAGCGAGTCATACCAGCAGGGCGTGCTGTCTGC
CACCATCCTGTATGAGATCCTGCTCGGCAAGGCCACACTGTACGCTGTGCTGGTGTCTGCTCT
GGTGCTGATGGCTATGGTCTCCCGGGAGCGCATCCCCGAGGCC

TCR-beta/constant (SEQ ID NO:96)

CGGGCCAAGCGG

Furin cleavage site (SEQ ID NO:97)

GGCAGCGGC

GSG Linker (SEQ ID NO:98)

GCCACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACGTGGAGGAGAACCCCGGCCCT

P2A (SEQ ID NO:99)

ATGGCCACAGGCAGCAGAACATCTCTGCTGCTGGCCTTCGGACTGCTGTGTCTGCCTTGGCT
GCAAGAGGGTTCCGCC

HGH/SS/2 (SEQ ID NO:100)

[[TRA_VDJ Sequence, specific for the NeoTCR]]

Insertion site for neoTCR TRA_VDJ sequence

ATATTCAGAACCCCGATCCTGCTGTATCAGCTGCGCGACAGCAAGAGCAGCGACAAGAGC
GTGTGTTTGTTC

TCR-alpha/constant (SEQ ID NO:102)

FIGURE 8 Cont.

TRAC CDS/right HR arm (SEQ ID NO:103)

ACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGAC
AAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCA
ACAAATCTGACTTTGCATGTGCAAACGCCTTCAACACAGCATTATTCCAGAAGACACCTTCT
TCCCCAGCCCAGG

Right HR arm (SEQ ID NO:104)

TAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAATGGCCAGGTTCTGCC
CAGAGCTCTGGTCAATGATGTCTAAAACTCCTCTGATTGGTGGTCTCGGCCTTATCCATTGCC
ACCAAAACCCTCTTTTTACTAAGAAACAGTGAGCCTTGTTCTGGCAGTCCAGAGAATGACAC
GGGAAAAAAGCAGATGAAGAAGAGGCAGGAGAGGGCACGTGGCCCAGCCTCAGTCT
CTCCAACTGAGTTCCTGCCTGCCTGCCTTTGCTCAGACTGTTTGCCCCTTACTGCTCTTCTAGG
CCTCATTCTAAGCCCCTTCTCCAAGTTGCCTCTCCTTATTTCTCCCTGTCTGCCAAAAAATCTTT
CCCAGCTCACTAAGTCAGTCTCACGCAGTCACTCATTAACCCACCAATCACTGATTGTGCCGG
CACATGAATGCACCAGGTGTTGAAGTGGAGGAATTAAAAAGTCAGATGAGGGGTGTGCCCA
GAGGAAGCACCATTCTAGTTGGGGGAGCCCATCTGTCAGCTGGGAAAAGTCCAAATAACTT
CAGATTGGAATGTGTTTTAACTCAGGGTTGAGAAAACAGCTACCTTCAGGACAAAAGTCAG
GGAAGGGCTCTCTGAAGAAATGCTACTTGAAGATACCAGCCCTACCAAGGGCAGGGAGAG
GACCCTATAGAGGCCTGGGACAGGAGCTCAATGAGAAAGGAGAAGAGCAGCAGGCATGA
GTTGAATGAAGGAGGCAGGGCCGGGTCACAGGGCCTTCTAGGCCATGAGAGGGGTAGACAG

(SEQ ID NO:105)

GCTAGC

FIGURE 8 Cont.

pBR322_origin (SEQ ID NO:106)

CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAG
CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCT
TCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT
CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGG
TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTG
GTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCC
TAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTT
CGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCT

TTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCA
TATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGAT
GGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTC
CCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGA
GAATGGCAAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTC
ATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCCAGACGAAA
TACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAAC
ACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTG
TTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTG
ATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCA
TTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAAG
CGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAG
CATCCATGTTGGAATTTAATCGCGGCCTCGACGTTTCCCGTTGAATATGGCTCAT

AACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATC
TTGTGCAATGTAACATCAGAGATTTTGAGACAC

Kanamycin resistance (KanR2) (SEQ ID NO:107)

Kanamycin promoter (SEQ ID NO:108)

FIGURE 9

CD8 Product 4 Product Nucleic Acid Sequence

Left HR Arm (SEQ ID NO:110)

ACATTAAAAACACAAAATCCTACGGAAATACTGAAGAATGAGTCTCAGCACTAAGGAAAAGC
CTCCAGCAGCTCCTGCTTTCTGAGGGTGAAGGATAGACGCTGTGGCTCTGCATGACTCACTA
GCACTCTATCACGGCCATATTCTGGCAGGGTCAGTGGCTCCAACTAACATTTGTTTGGTACTT
TACAGTTTATTAAATAGATGTTTATATGGAGAAGCTCTCATTTCTTTCTCAGAAGAGCCTGGCT
AGGAAGGTGGATGAGGCACCATATTCATTTTGCAGGTGAAATTCCTGAGATGTAAGGAGCT
GCTGTGACTTGCTCAAGGCCTTATATCGAGTAAACGGTAGTGCTGGGGCTTAGACGCAGGTG
TTCTGATTTATAGTTCAAAACCTCTATCAATGAGAGAGCAATCTCCTGGTAATGTGATAGATTT
CCCAACTTAATGCCAACATACCATAAACCTCCCATTCTGCTAATGCCCAGCCTAAGTTGGGGA
GACCACTCCAGATTCCAAGATGTACAGTTTGCTTTGCTGGGCCTTTTTCCCATGCCTGCCTTTA
CTCTGCCAGAGTTATATTGCTGGGGTTTTGAAGAAGATCCTATTAAATAAAAGAATAAGCAGT
ATTATTAAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACG
TTCACTGAAATCATGGCCTCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTC
CATCACGAGCAGCTGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTGCC
AGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTCCAGCCTGGGTTGGGGCA
AAGAGGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAG

TRAC CDS (SEQ ID NO:111)

ATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTG
TCTGCCTATTC

GSG Linker 1 (SEQ ID NO:112)

GAATTCGGCTCCGGA

P2A (SEQ ID NO:113)

GCCACTAACTTCAGCCTGTTGAAGCAGGCCGGCGACGTTGAGGAAAACCCCGGTCCT

FIGURE 9 Cont.

ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGGCC
G

CD8A Signal peptide (SEQ ID NO:114)

AGCCAGTTCCGGGTGTCGCCGCTGGATCGGACCTGGAACCTGGGCGAGACAGTGGAGCTG
AAGTGCCAGGTGCTGCTGTCCAACCCGACGTCGGGCTGCTCGTGGCTCTTCCAGCCGCGCG
GCGCCGCCGCCAGTCCCACCTTCCTCCTATACCTCTCCCAAAACAAGCCCAAGGCGGCCGAG
GGGCTGGACACCCAGCGGTTCTCGGGCAAGAGGTTGGGGGACACCTTCGTCCTCACCCTG
AGCGACTTCCGCCGAGAGAACGAGGGCTACTATTTCTGCTCGGCCCTGAGCAACTCCATCAT
GTACTTCAGCCACTTCGTGCCGGTCTTCCTGCCAGCGAAGCCCACCACGACGCCAGCGCCGC
GACCACCAACACCGGCGCCCACCATCGCGTCGCAGCCCCTGTCCCTGCGCCCAGAGGCGTG
CCGGCCAGCGGCGGGGGGCGCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGAT

CD8A Extracellular domain (SEQ ID NO:115)

CD8A transmembrane domain (SEQ ID NO:116)

ATCTACATCTGGGCGCCCTTGGCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTTATCACC

CD4 Intracellular domain (SEQ ID NO:117)

TGTGTCAGGTGCCGGCACCGAAGGCGCCAAGCAGAGCGGATGTCTCAGATCAAGAGACTCC
TCAGTGAGAAGAAGACCTGCCAGTGTCCTCACCGGTTTCAGAAGACATGTAGCCCCATT

Furin cleavage site (SEQ ID NO:118)

AGGGCTAAACGG

GSG Linker (SEQ ID NO:119)

GAATTCGGCTCCGGA

P2A (SEQ ID NO:120)

GCCACTAACTTCTCCCTGTTGAAACAGGCTGGCGATGTTGAAGAAAACCCCGGTCCT

HGH/SS/2 (SEQ ID NO:121)

ATGGCCACCGGCTCTAGAACAAGCCTGCTCGCTTTTTGGCCTGCTCTGCCTCCCATGGCTC
CAAGAAGGATCTGCT

FIGURE 9 Cont.

[[TRB_VDJ Sequence, specific for the NeoTCR]]

Insertion site for neoTCR TRB_VDJ sequence

CTGAAAAACGTGTTCCCTCCAAAAGTGGCCGTGTTCGAGCCTTCTGAGGCCGAGATCAGCC
ACACACAGAAAGCCACACTCGTGTGTCTGGCTACCGGCTTCTACCCCGATCACGTGGAACTG
TCTTGGTGGGTCAACGGCAAAGAGGTGCACAGCGGCGTCAGCACAGATCCCCAGCCTCTGA
AAGAACAGCCCGCTCTGAACGACAGCCGCTACTGCCTGTCTAGCAGACTGAGAGTGTCCGC
CACCTTCTGGCAGAACCCCAGAAACCACTTCAGATGCCAGGTCCAGTTCTACGGCCTGAGCG
AGAACGATGAGTGGACCCAGGACAGAGCCAAGCCTGTGACACAGATCGTGTCTGCCGAAG
CCTGGGGCAGAGCCGATTGTGGCTTTACCAGCGAGTCATACCAGCAGGGCGTGCTGTCTGC
CACCATCCTGTATGAGATCCTGCTCGGCAAGGCCACACTGTACGCTGTGCTGGTGTCTGCTCT
GGTGCTGATGGCTATGGTCTCCCGGGAGCGCATCCCCGAGGCC

TCR-beta/constant (SEQ ID NO:123)

CGGGCCAAGCGG

Furin cleavage site (SEQ ID NO:124)

GGCAGCGGC

GSG Linker (SEQ ID NO:125)

GCCACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACGTGGAGGAGAACCCCGGCCCT

P2A (SEQ ID NO:126)

ATGGCCACAGGCAGCAGAACATCTCTGCTGCTGGCCTTCGGACTGCTGTGTCTGCCTTGGCT
GCAAGAGGGTTCCGCC

HGH/SS/2 (SEQ ID NO:127)

[[TRA_VDJ Sequence, specific for the NeoTCR]]

Insertion site for neoTCR TRA_VDJ sequence

ATATTCAGAACCCCGATCCTGCTGTGTATCAGCTGCGCGACAGCAAGAGCAGCGACAAGAGC
GTGTGTTTGTTC

TCR-alpha/constant (SEQ ID NO:129)

SUBSTITUTE SHEET (RULE 26)

FIGURE 9 Cont.

TRAC CDS/right HR arm (SEQ ID NO:130)

ACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGAC
AAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCA
ACAAATCTGACTTTGCATGTGCAAACGCCTTCAACACAGCATTATTCCAGAAGACACCTTCT
TCCCCAGCCCAGG

Right HR arm (SEQ ID NO:131)

TAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAATGGCCAGGTTCTGCC
CAGAGCTCTGGTCAATGATGTCTAAAACTCCTCTGATTGGTGGTCTCGGCCTTATCCATTGCC
ACCAAAACCCTCTTTTTACTAAGAAACAGTGAGCCTTGTTCTGGCAGTCCAGAGAATGACAC
GGGAAAAAAGCAGATGAAGAGAAGGTGGCAGGAGAGGGCACGTGGCCCAGCCTCAGTCT
CTCCAACTGAGTTCCTGCCTGCCTTTGCTCAGACTGTTTGCCCCTTACTGCTCTTCTAGG
CCTCATTCTAAGCCCCTTCTCCAAGTTGCCTCTCCTTATTTCTCCCTGTCTGCCAAAAAATCTTT
CCCAGCTCACTAAGTCAGTCTCACGCAGTCACTCATTAACCCACCAATCACTGATTGTGCCGG
CACATGAATGCACCAGGTGTTGAAGTGGAGGAATTAAAAAGTCAGATGAGGGGTGTGCCCA
GAGGAAGCACCATTCTAGTTGGGGGAGCCCATCTGTCAGCTGGGAAAAGTCCAAATAACTT
CAGATTGGAATGTGTTTTAACTCAGGGTTGAGAAAACAGCTACCTTCAGGACAAAAGTCAG
GGAAGGGCTCTCTGAAGAAATGCTACTTGAAGATACCAGCCCTACCAAGGGCAGGGAGAG
GACCCTATAGAGGCCTGGGACAGGAGCTCAATGAGAAAGGAGAAGAGCAGCAGGCATGA
GTTGAATGAAGGAGGCAGGGCCGGGTCACAGGGCCTTCTAGGCCATGAGAGGGTAGACAG

(SEQ ID NO:132)

GCTAGC

FIGURE 9 Cont.

pBR322_origin (SEQ ID NO:133)

CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAG
CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCT
TCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTT
CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGG
TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTG
GTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCC
TAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTT
CGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCT

TTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCA
TATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGAT
GGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTC
CCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGA
GAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTC
ATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCCAGACGAAA
TACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAAC
ACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTG
TTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTG
ATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCA
TTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAAG
CGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAG
CATCCATGTTGGAATTTAATCGCGGCCTCGACGTTTCCCGTTGAATATGGCTCAT

AACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATC
TTGTGCAATGTAACATCAGAGATTTTGAGACAC

Kanamycin resistance (KanR2) (SEQ ID NO:134)

Kanamycin promoter (SEQ ID NO:135)

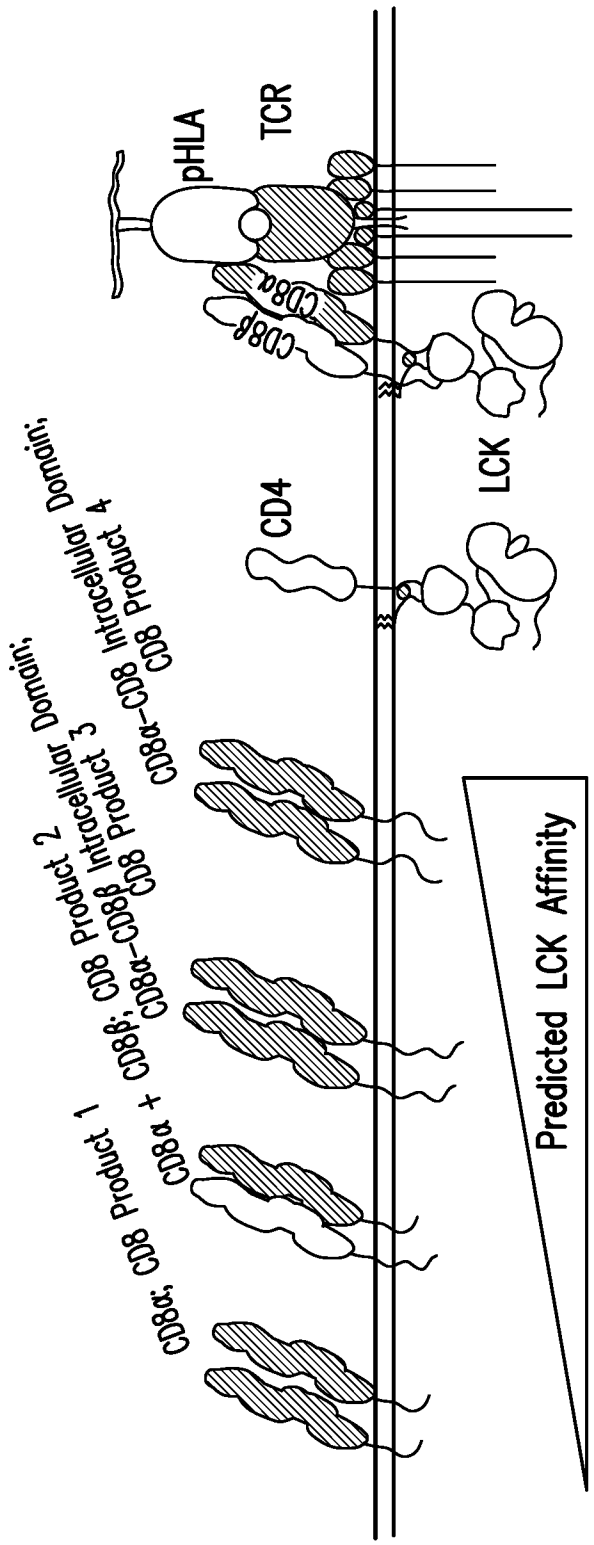

pHLA
TCR
CD8α
CD8β
CD4
LCK
CD8α; CD8 Product 1
CD8α + CD8β; CD8 Product 2
CD8α–CD8β Intracellular Domain; CD8 Product 3
CD8α–CD8 Intracellular Domain; CD8 Product 4
Predicted LCK Affinity

FIGURE 10

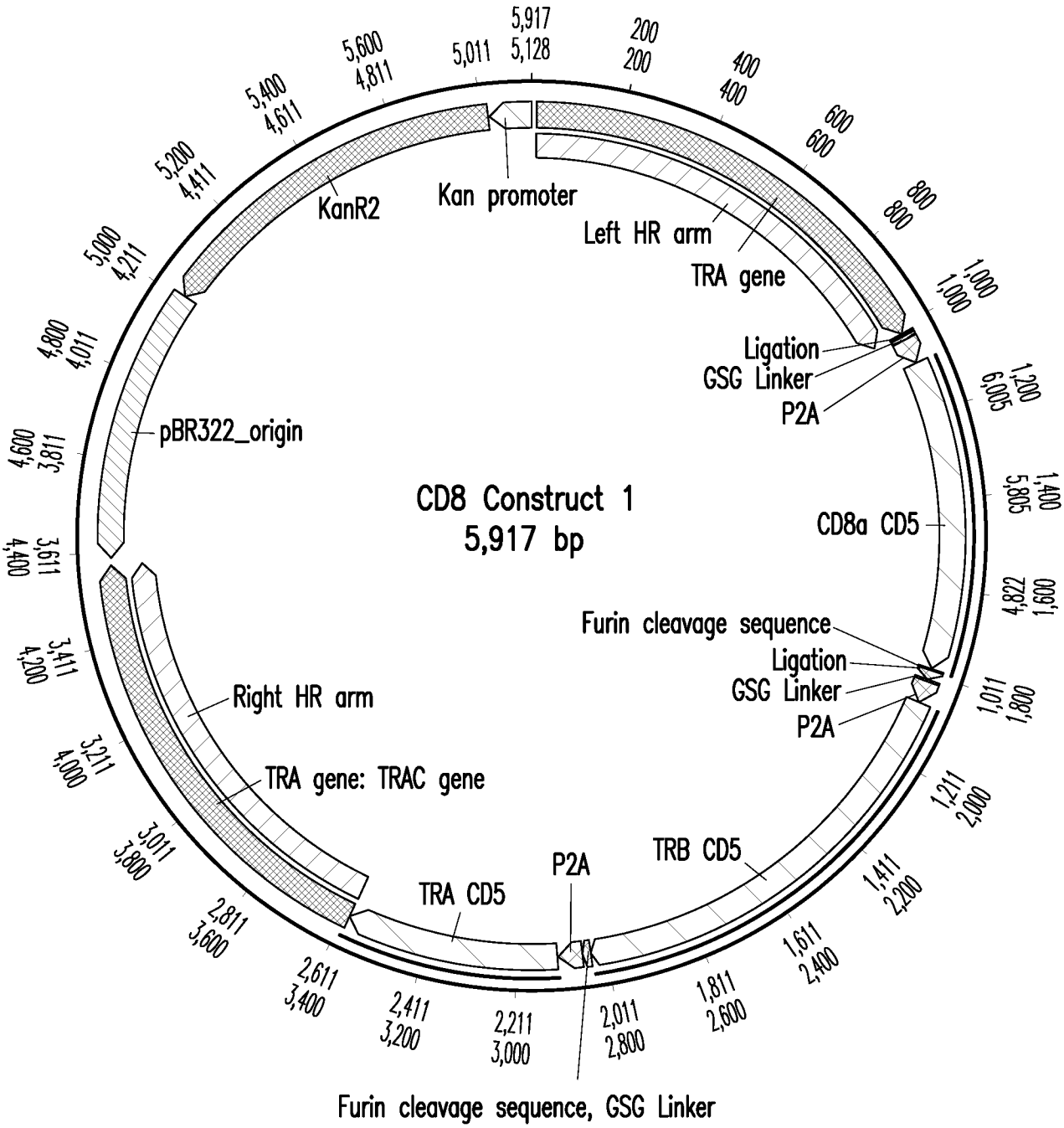

CD8 Construct 1
5,917 bp
KanR2
Kan promoter
Left HR arm
TRA gene
Ligation
GSG Linker
P2A
CD8a CD5
Furin cleavage sequence
Ligation
GSG Linker
P2A
TRB CD5
P2A
TRA CD5
TRA gene: TRAC gene
Right HR arm
pBR322_origin
Furin cleavage sequence, GSG Linker

FIGURE 11A

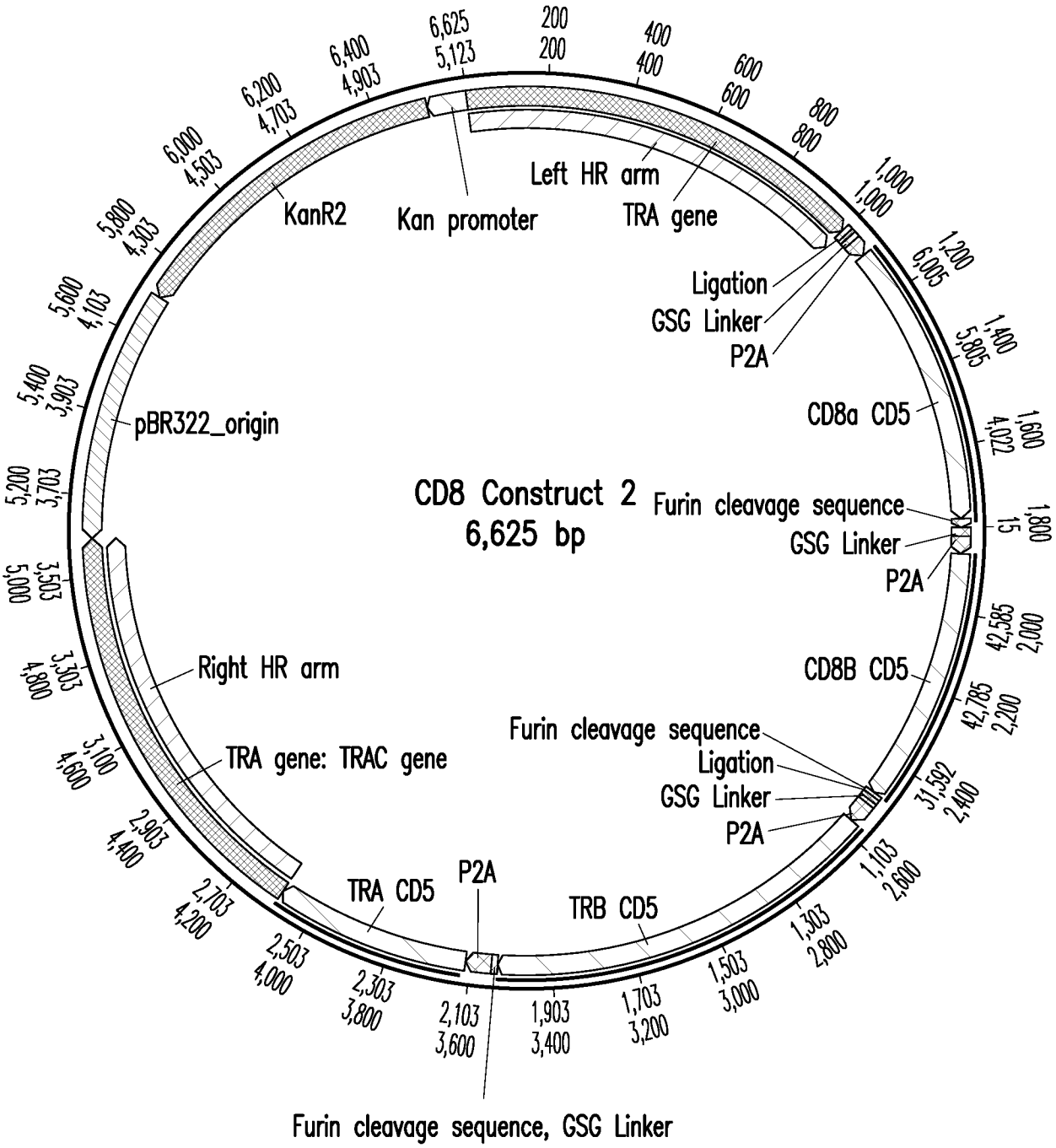

CD8 Construct 2
6,625 bp
Left HR arm
TRA gene
Ligation
GSG Linker
P2A
CD8a CD5
Furin cleavage sequence
GSG Linker
P2A
CD8B CD5
Furin cleavage sequence
Ligation
GSG Linker
P2A
TRB CD5
P2A
TRA CD5
TRA gene: TRAC gene
Right HR arm
pBR322_origin
KanR2
Kan promoter
Furin cleavage sequence, GSG Linker

FIGURE 11B

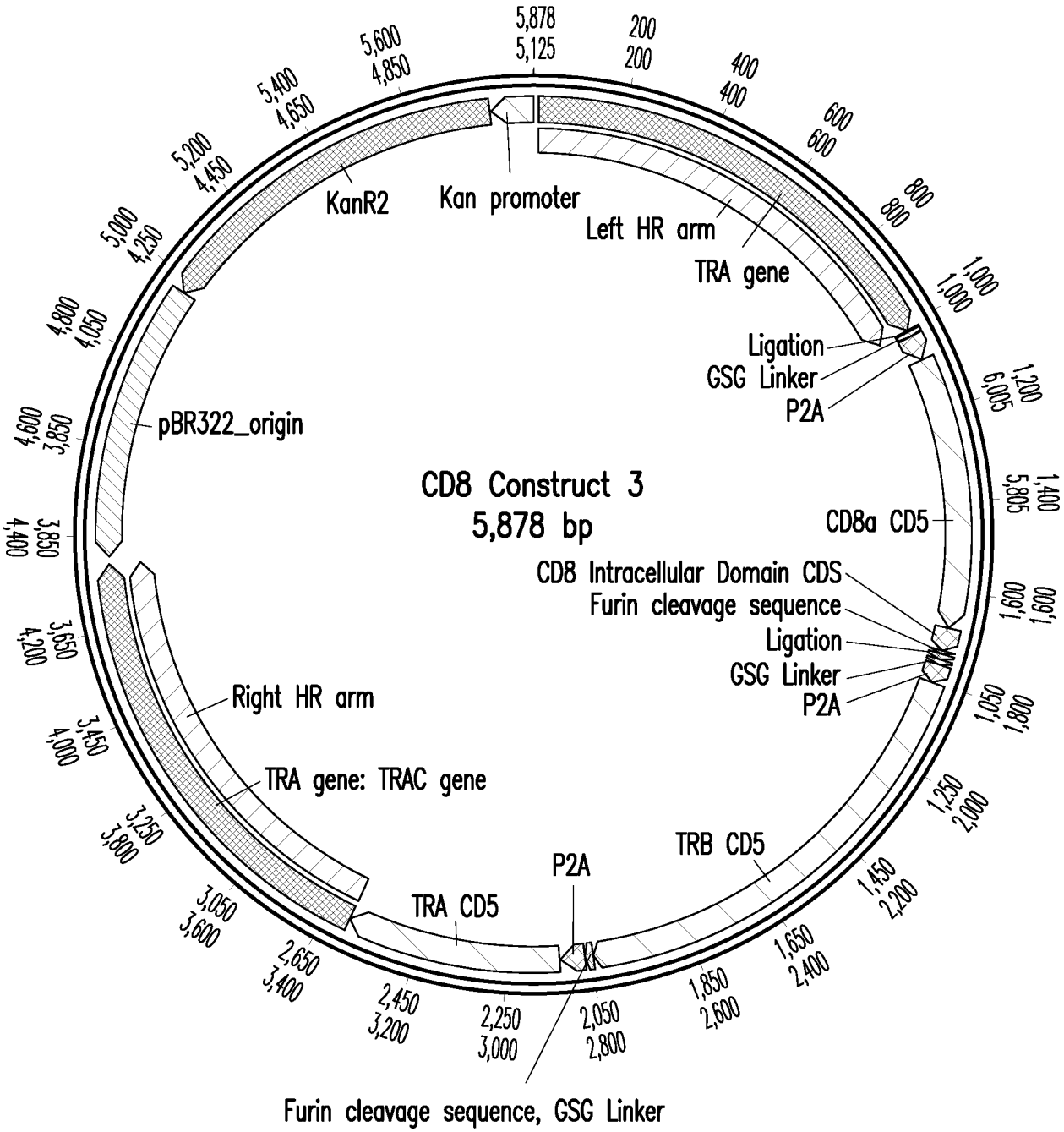

CD8 Construct 3
5,878 bp
KanR2
Kan promoter
Left HR arm
TRA gene
Ligation
GSG Linker
P2A
CD8a CD5
CD8 Intracellular Domain CDS
Furin cleavage sequence
Ligation
GSG Linker
P2A
TRB CD5
P2A
TRA CD5
TRA gene: TRAC gene
Right HR arm
pBR322_origin
Furin cleavage sequence, GSG Linker

FIGURE 11C

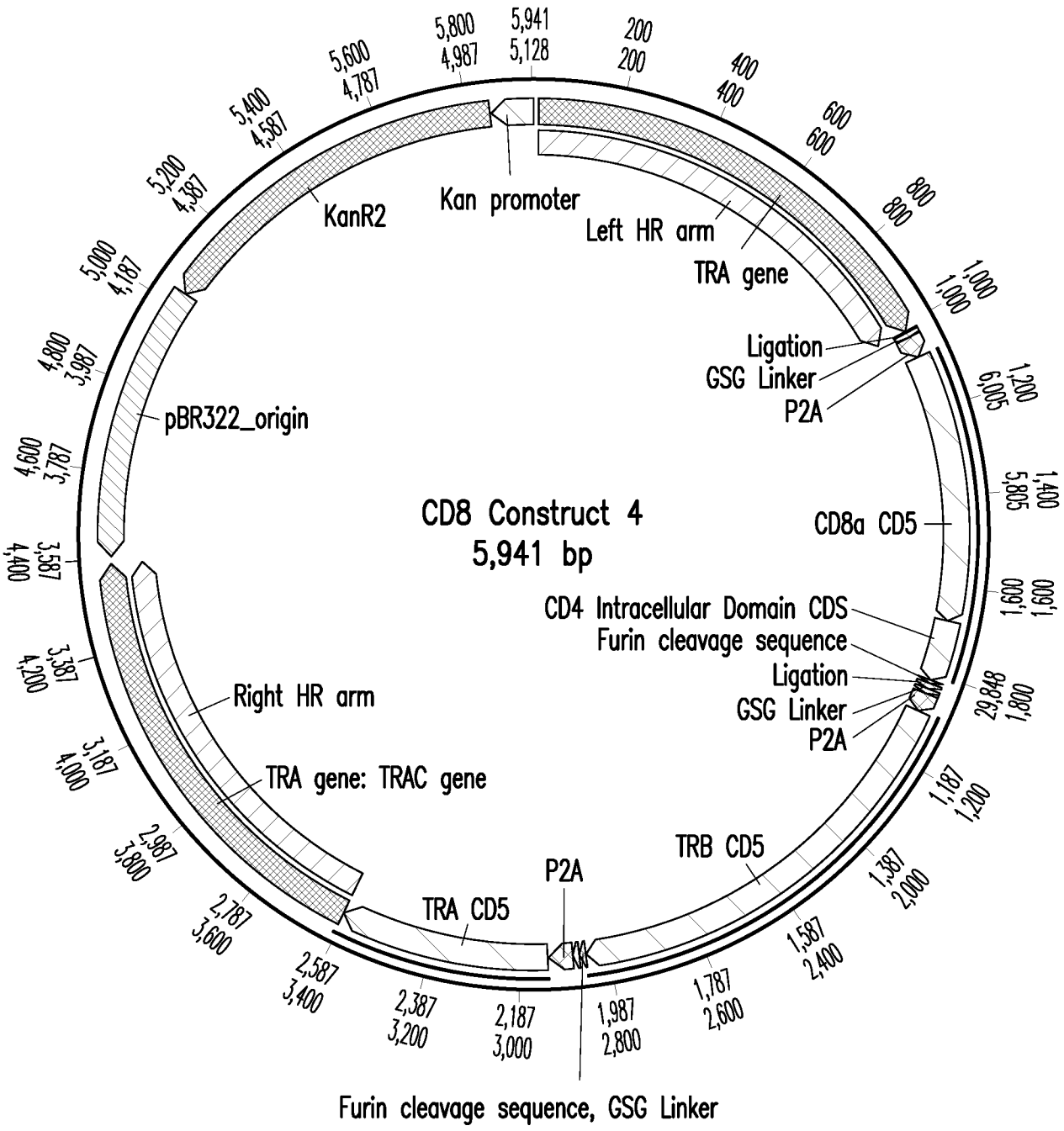

CD8 Construct 4
5,941 bp
Kan promoter
KanR2
Left HR arm
TRA gene
Ligation
GSG Linker
P2A
CD8a CD5
CD4 Intracellular Domain CDS
Furin cleavage sequence
Ligation
GSG Linker
P2A
TRB CD5
P2A
TRA CD5
TRA gene: TRAC gene
Right HR arm
pBR322_origin
Furin cleavage sequence, GSG Linker

FIGURE 11D

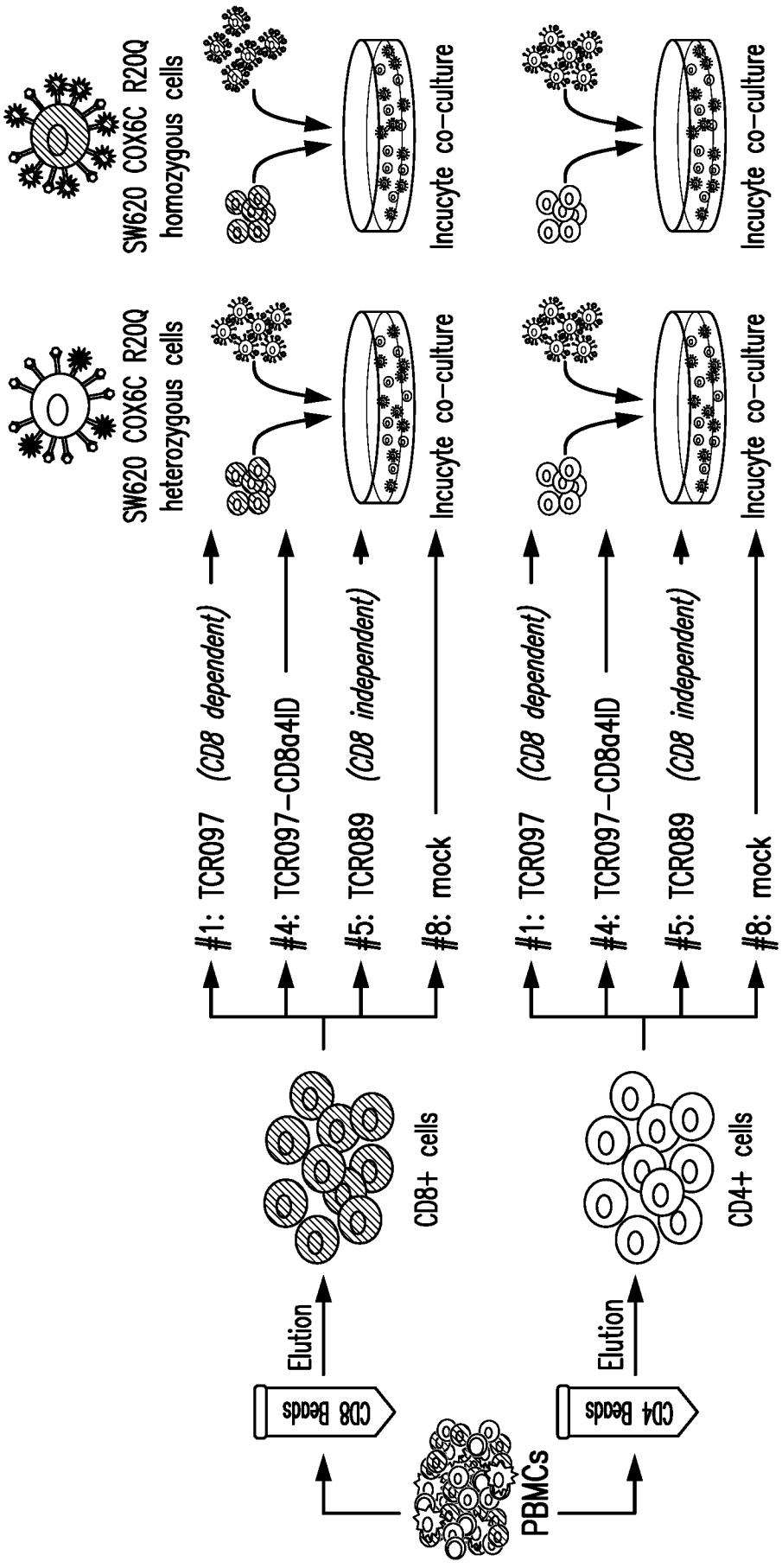

PBMCs
CD8 Beads
CD4 Beads
Elution
Elution
CD8+ cells
CD4+ cells
#1: TCR097 (CD8 dependent)
#4: TCR097-CD8a4ID
#5: TCR089 (CD8 independent)
#8: mock
#1: TCR097 (CD8 dependent)
#4: TCR097-CD8a4ID
#5: TCR089 (CD8 independent)
#8: mock
Incucyte co-culture
Incucyte co-culture
Incucyte co-culture
Incucyte co-culture
SW620 COX6C R20Q heterozygous cells
SW620 COX6C R20Q homozygous cells

FIGURE 12

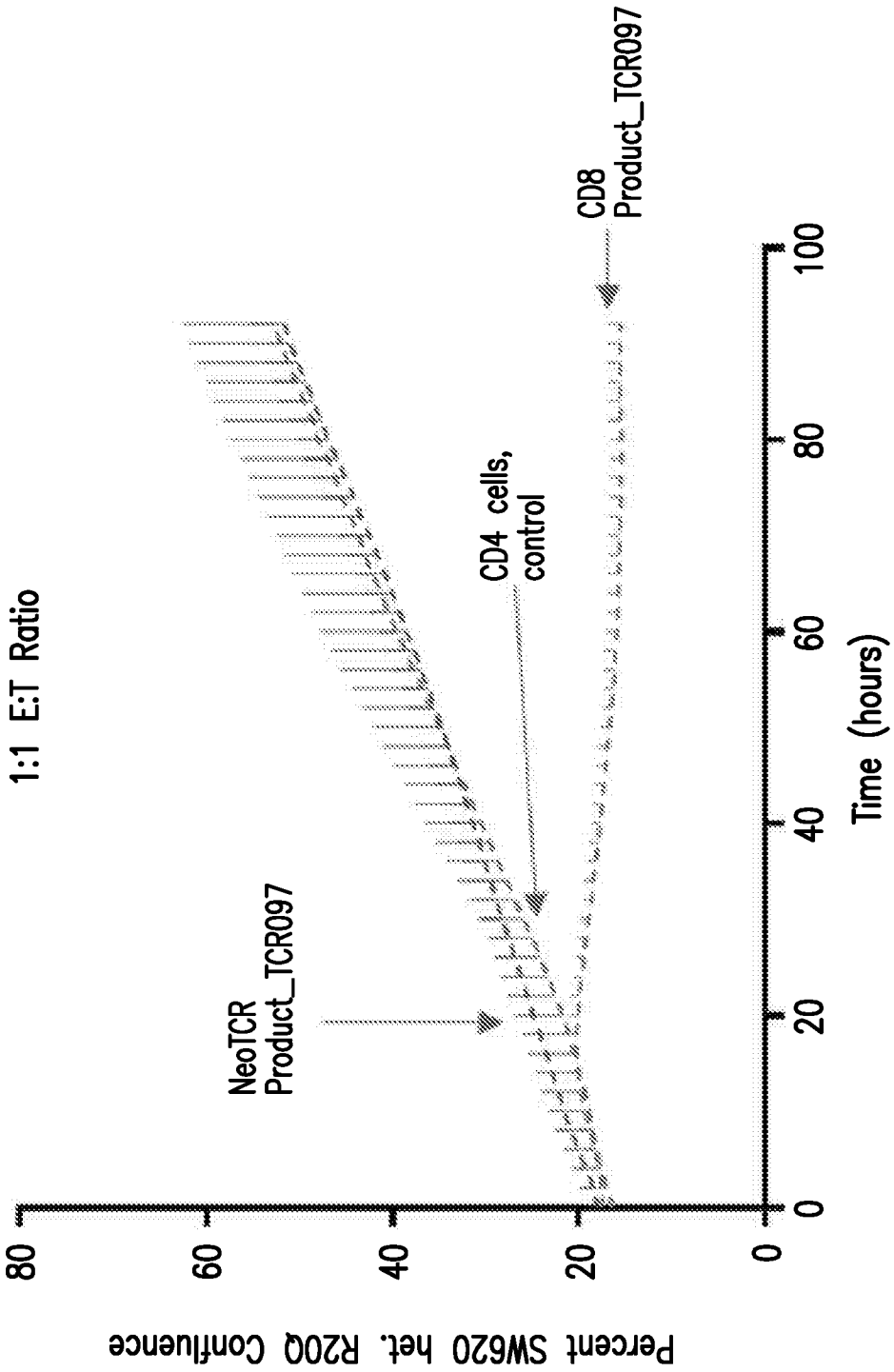

1:1 E:T Ratio
NeoTCR Product_TCR097
CD4 cells, control
CD8 Product_TCR097
Percent SW620 het. R200Q Confluence
Time (hours)
0
20
40
60
80
0
20
40
60
80
100

FIGURE 13A

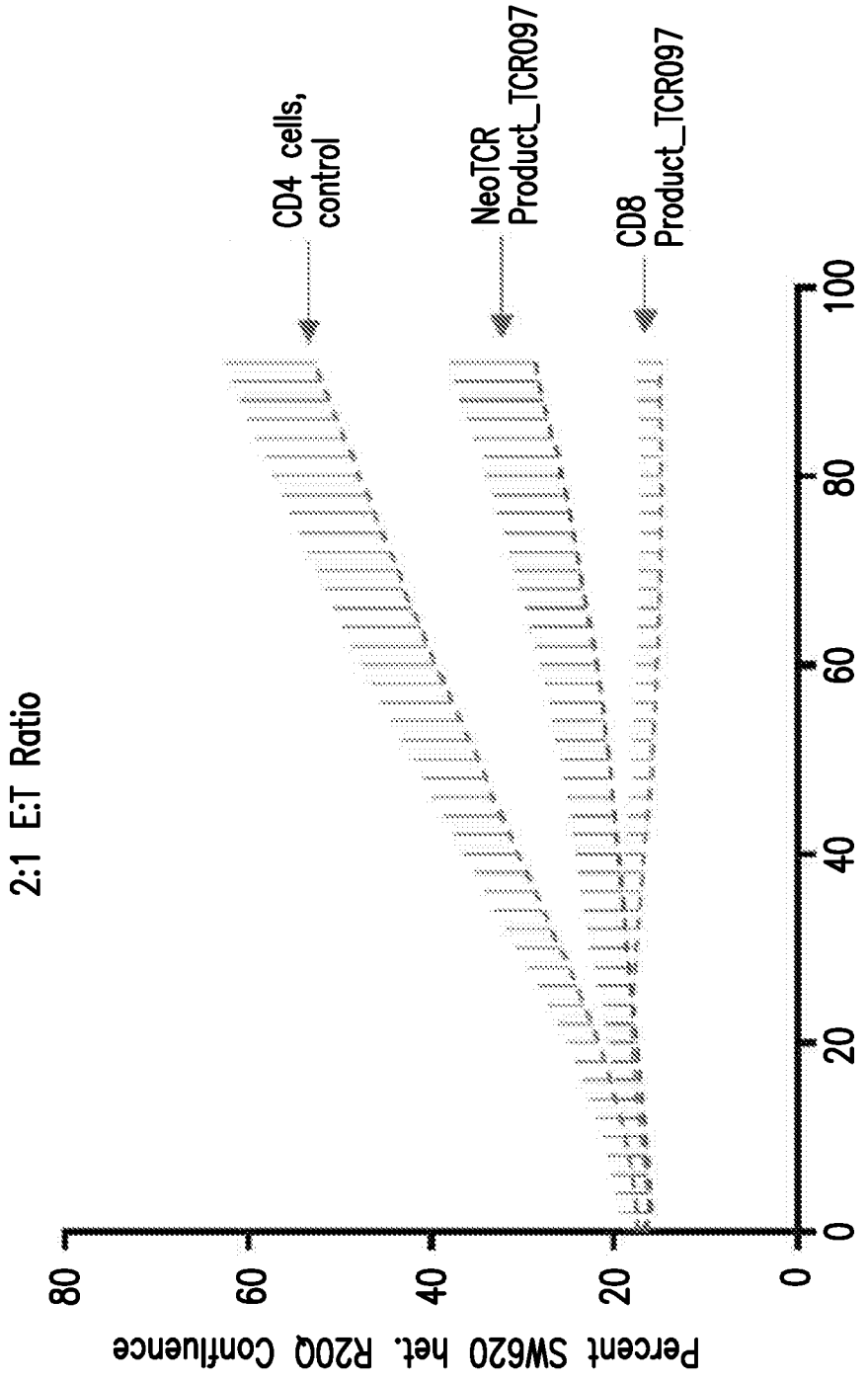

CD4 cells, control
NeoTCR Product_TCR097
CD8 Product_TCR097
2:1 E:T Ratio
Percent SW620 het. R200Q Confluence
0
20
40
60
80
0
20
40
60
80
100
Time (hours)

FIGURE 13B

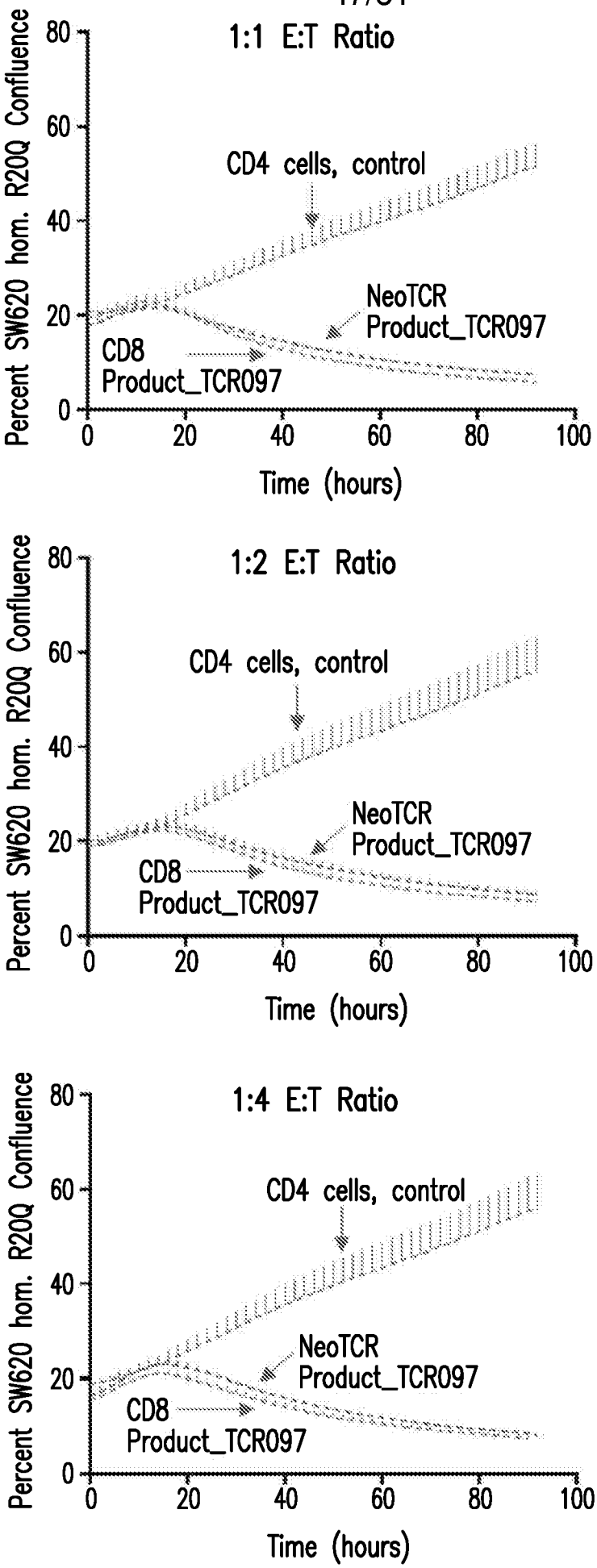
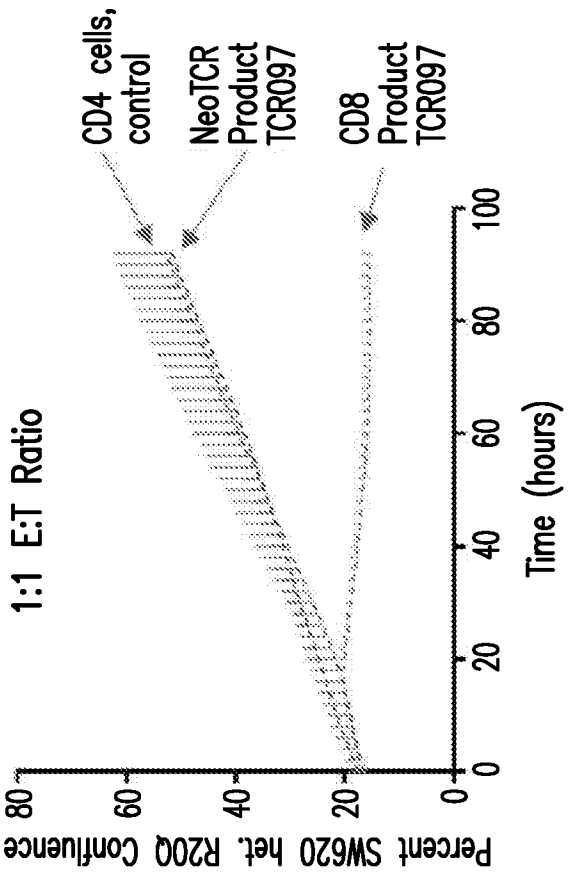

1:1 E:T Ratio
Percent SW620 hom. R20Q Confluence
CD4 cells, control
NeoTCR
Product_TCR097
CD8
Product_TCR097
Time (hours)
1:2 E:T Ratio
Percent SW620 hom. R20Q Confluence
CD4 cells, control
NeoTCR
Product_TCR097
CD8
Product_TCR097
Time (hours)
1:4 E:T Ratio
Percent SW620 hom. R20Q Confluence
CD4 cells, control
NeoTCR
Product_TCR097
CD8
Product_TCR097
Time (hours)

FIGURE 14



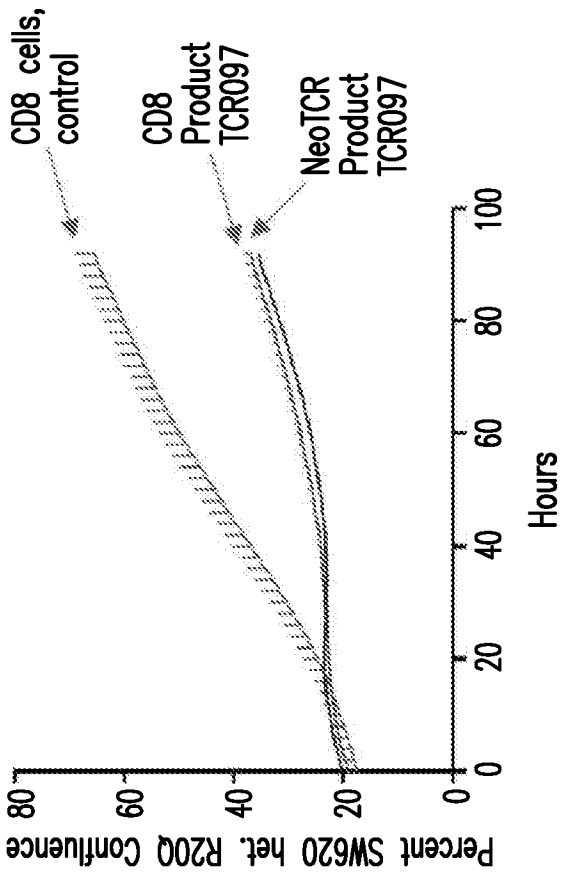
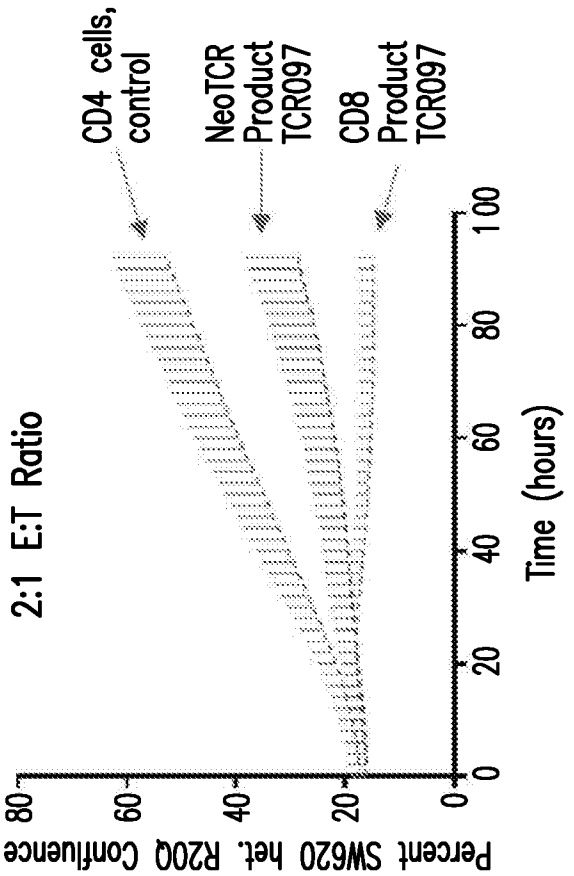


FIGURE 15B



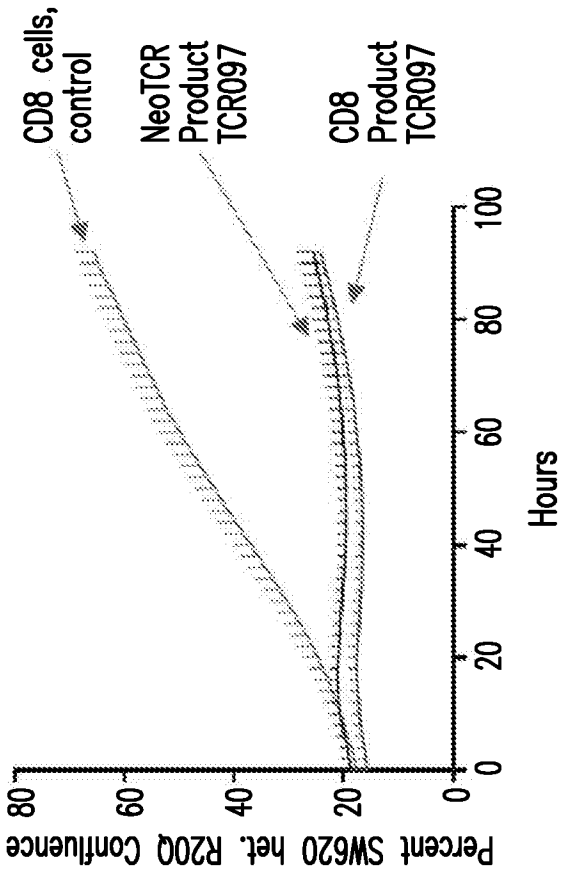


FIGURE 15A

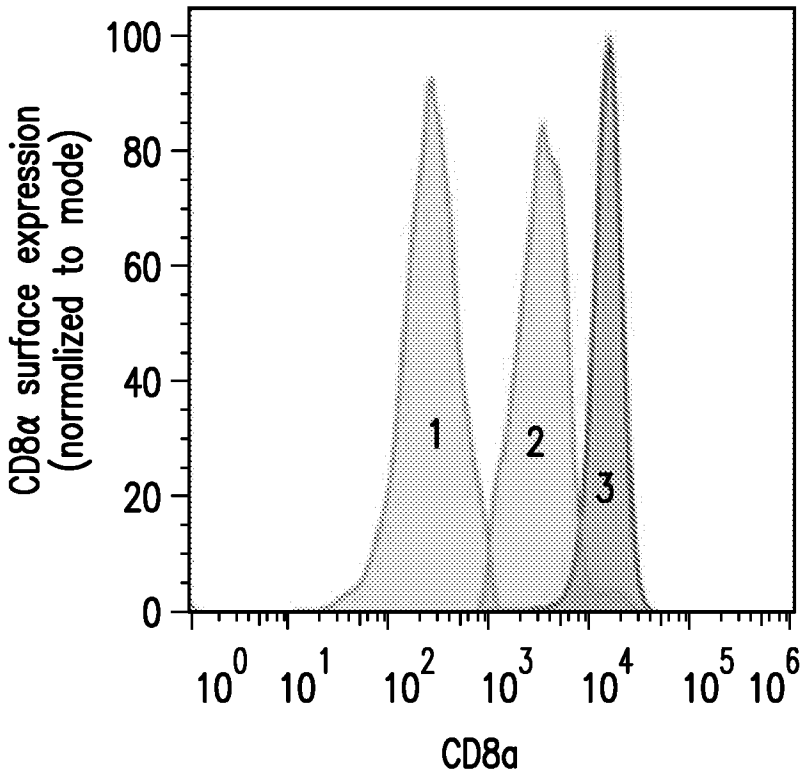

CD8α surface expression
(normalized to mode)
100
80
60
40
20
0
1
2
3
10^0 10^1 10^2 10^3 10^4 10^5 10^6
CD8α

FIGURE 16

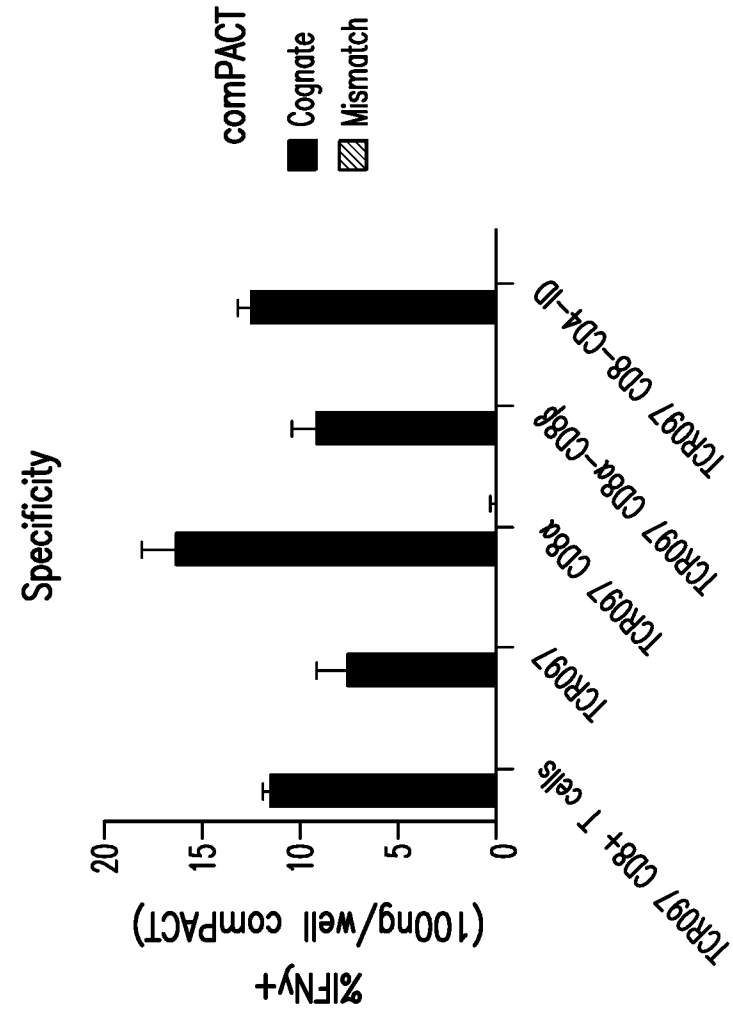


FIGURE 17B

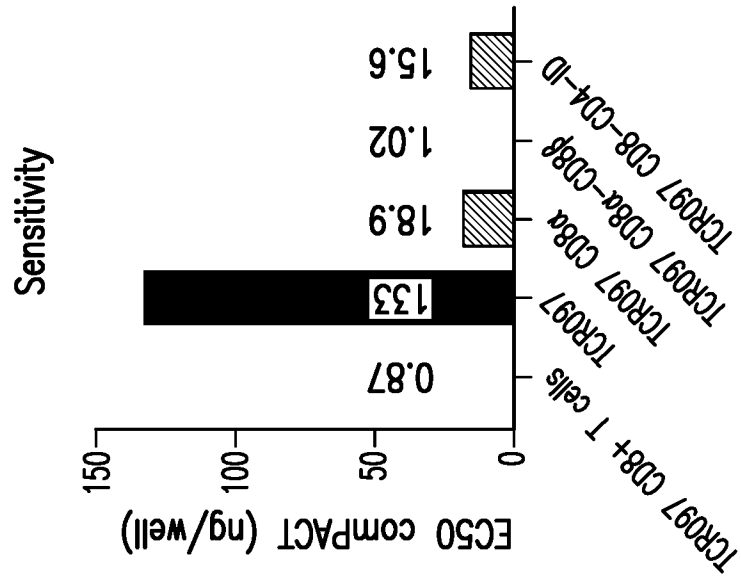


FIGURE 17A

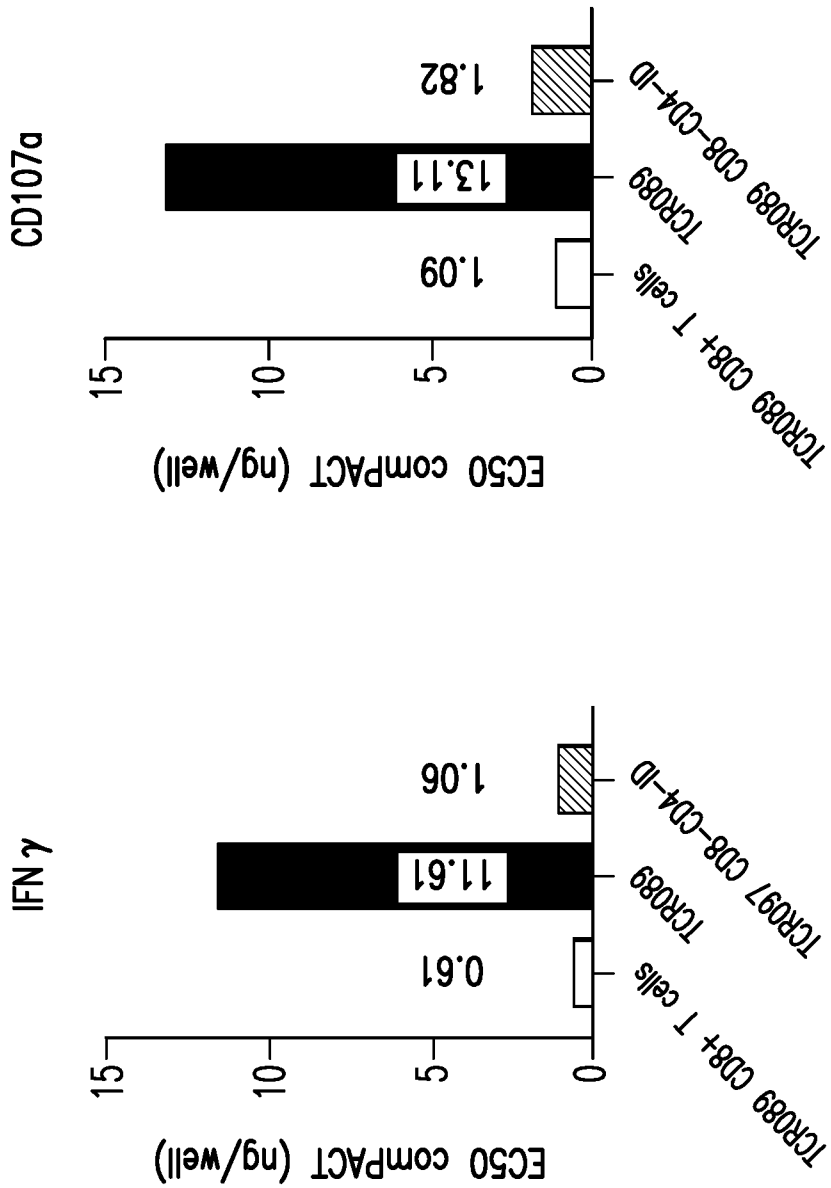

CD107a
EC50 comPACT (ng/well)
0
5
10
15
1.09
13.11
1.82
TCR089 CD8+ T cells
TCR089
TCR089 CD8-CD4-ID
IFNγ
EC50 comPACT (ng/well)
0
5
10
15
0.61
11.61
1.06
TCR089 CD8+ T cells
TCR089
TCR097 CD8-CD4-ID

FIGURE 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/030818

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 14/725; C07K 14/73; C07K 16/28; C07K 16/32; C12N 5/0783 (2020.01)
CPC - C07K 16/2803; C07K 16/2809; C07K 2317/622; C07K 2319/03 (2020.05)

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2018/170338 A2 (FRED HUTCHINSON CANCER RESEARCH CENTER) 20 September 2018 (20.09.2018) entire document	1-5, 123, 127, 129 ---------- 48, 124-126, 128, 130
Y	US 6,355,412 B1 (STEWART et al) 12 March 2002 (12.03.2002) entire document	48, 125, 126
Y	US 2004/0265970 A1 (GEARING et al) 30 December 2004 (30.12.2004) entire document	124, 126, 128, 130
Y	US 2017/0319722 A1 (INDI MOLECULAR, INC.) 09 November 2017 (09.11.2017) entire document	124, 126, 128, 130
Y	US 2015/0110760 A1 (ZHANG et al) 23 April 2015 (23.04.2015) entire document	124, 126, 128, 130
Y	US 2018/0185463 A1 (ALLOPLEX BIOTHERAPEUTICS) 05 July 2018 (05.07.2018) entire document	124, 126, 128, 130
P, X	WO 2020/049496 A1 (GLAXOSMITHKLINE INTELLECTUAL PROPERTY DEVELOPMENT LIMITED et al) 12 March 2020 (12.03.2020) entire document	1-5, 48-50, 123-130
P, X	WO 2019/089610 A1 (PACT PHARMA, INC.) 09 May 2019 (09.05.2019) entire document	1-5, 48-50, 123-130

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

Date of the actual completion of the international search	Date of mailing of the international search report
30 July 2020	14 AUG 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/030818

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

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

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

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

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

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

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

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

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

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

3. Additional comments:

SEQ ID NOs: 139-148 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/030818

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

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

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

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

3. ☒ Claims Nos.: 6-47, 51-122
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.