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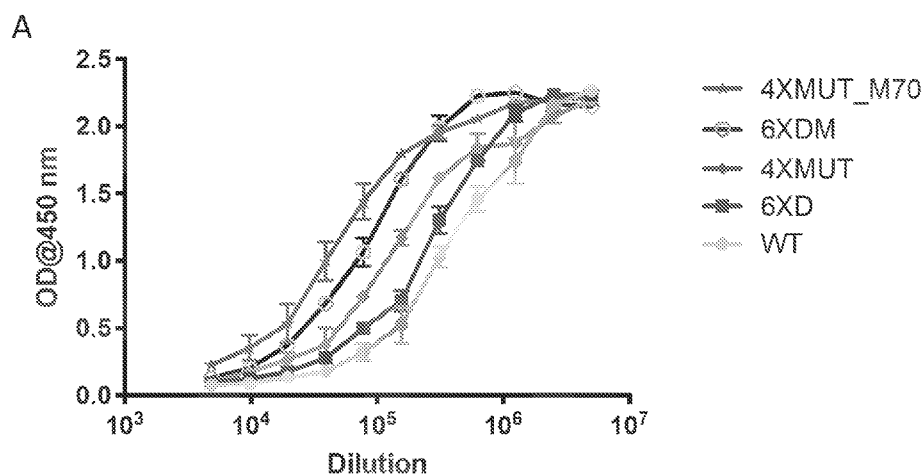


FIG. 1A

(57) Abstract: The present disclosure relates to novel PD-1 decoy variants, compositions, and methods to confer and/or increase immune responses mediated by cellular immunotherapy, such as by adoptively transferring tumor-specific genetically-modified subsets of lymphocytes.

PD-1 DECOY VARIANTS FOR IMMUNOTHERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Patent
5 Application No. 63/113,651, filed November 13, 2020. The foregoing application is incorporated
by reference herein in its entirety.

FIELD OF THE INVENTION

The present invention relates generally to PD-1 decoy variants, compositions, and methods
for immunotherapy.

BACKGROUND OF THE INVENTION

Adoptive cell transfer or adoptive cell therapy (ACT) represents a promising therapeutic
approach for the treatment of cancer patients. However, it faces two major obstacles: the short-
term survival of the transferred cells in the cancer patients and the hostile immunosuppressive
tumor microenvironment.

To overcome these limitations, several options have been proposed. For example, some
15 trials tested the administration of interleukin 2 (IL-2) concurrently with the ACT. IL-2 is a potent
immunostimulant; therefore it boosts the immune response and increases the survival of the
transferred cells. However, this approach was unsuccessful due to the toxicities associated with
IL-2. US Patent 7,381,405 describes methods for preparing IL-2-transduced lymphocytes for ACT
20 that secrete IL-2. This approach is based on the hypothesis that the lymphocytes will secrete their
own growth factor (*e.g.*, IL-2) and thus depend less on other exogenous factors for survival *in vivo*.
Despite the successful results in *in vitro* settings, clinical trials determined that this approach was
ineffective. IL-2-transduced lymphocytes were not more effective than non-transduced
lymphocytes in treating cancer (Heemskerk *et al.*, Human Gene Therapy, 2008).

The advent of chimeric antigen receptor (CAR) T cells has provided a useful tool to
25 improve ACT. TRUCKs (International Publication WO 2017/108805) and Armored CARs (US
Patent 10,124,023) are representative examples of CAR T cells that have been further engineered
for the secretion of a recombinant interleukin-12 (IL-12) and CD40L, respectively. However, these
strategies have disadvantages. For example, in TRUCKs, high transgenic IL-12 production limited

T cell expansion and increased apoptosis, showing limited therapeutic efficacy. Also, the clinical application of armored CAR T cells has been limited to liquid tumors so far.

The solid tumors and their microenvironment have given a series of challenges for the success of ACT therapy. These challenges include efficient trafficking and infiltration of the tumor, as well as overcoming tumor-mediated immunosuppression. Despite numerous efforts, the state-of-the-art ACT therapies do not provide functional persistence within the immunosuppressive solid tumor microenvironment for long-term efficacy.

Therefore, there is a pressing need for identifying novel ACT therapies that provide cells with functional persistence and/or that can change the cytokine milieu to overcome the immunosuppressive tumor microenvironment.

SUMMARY OF THE INVENTION

This disclosure addresses the need mentioned above in a number of aspects. In one aspect, this disclosure provides a programmed cell death 1 (PD-1) decoy polypeptide. The PD-1 decoy polypeptide comprises an amino acid sequence of a PD-1 variant, wherein the amino acid sequence has at least 80% identity to SEQ ID NO: 6 and comprises substitutions at least at positions T76, K78, and Q133 of SEQ ID NO: 6. In some embodiments, the substitutions comprise additional substitutions at least at one of positions L122 and A132.

In some embodiments, the substitutions comprise: (i) T76D or a conservative substitution of D76; or T76V or a conservative substitution V76; (ii) K78R or a conservative substitution of R78; or (iii) Q133K or a conservative substitution of K133.

In some embodiments, the additional substitutions comprise at least one of: (i) L122E or a conservative substitution of E122; L122F or a conservative substitution of F122; or L122I or a conservative substitution of I122; and (ii) A132W or a conservative substitution of W132; A132L or a conservative substitution of L132; or A132Y or a conservative substitution of Y132.

In some embodiments, the amino acid sequence comprises the substitutions at the positions at T76, K78, E122, A132, and Q133. In some embodiments, the substitutions comprise: (a) T76D, K78R, L122F, and Q133K; or (b) T76V, K78R, L122F, A132W, and Q133K.

In some embodiments, the PD-1 decoy polypeptide further comprises a fusion partner. In some embodiments, the fusion partner comprises a fragment of a human immunoglobulin

polypeptide sequence. In some embodiments, the fragment comprises: (a) a CH3 domain; and (b) part or whole of an Fc region. In some embodiments, the fragment comprises a human IgG Fc sequence (*e.g.*, IgG4Fc).

5 In some embodiments, the PD-1 decoy polypeptide further comprises a cellular elimination tag (CET). In some embodiments, the CET comprises a truncated EGFR (tEGFR) or a variant thereof, a truncated HER2 (tHER2) or a variant thereof, a CD20 or a variant thereof, or a CD19 or a variant thereof.

10 In some embodiments, the fusion partner is linked (directly or indirectly via a linker) to the C-terminus of the amino acid sequence of the PD-1 variant, and the CET is linked to the C-terminus of the fusion partner.

In some embodiments, the PD-1 decoy polypeptide comprises an amino acid sequence selected from SEQ ID NOs: 9-65, 90, 92, 95-96, and 99-100.

15 In some embodiments, the tEGFR comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 88 or the amino acid sequence of SEQ ID NO: 88; the HER2 comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 93 or the amino acid sequence of SEQ ID NO: 93; and the CD20 comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 98 or the amino acid sequence of SEQ ID NO: 98.

In some embodiments, the PD-1 decoy polypeptide comprises a detectable label.

20 Also within the scope of this disclosure is a polynucleotide comprising a polynucleotide sequence that encodes the PD-1 decoy polypeptide of any one of the preceding claims. In some embodiments, the polynucleotide comprises: (a) a nucleotide sequence encoding an alpha polypeptide and a beta polypeptide of a T cell receptor (TCR); or (b) a nucleotide sequence encoding a chimeric antigen receptor (CAR). Also provided is a vector comprising the polynucleotide as described above.

25 In another aspect, the disclosure also provides a cell comprising the described polynucleotide. In some embodiments, the cell further comprises a second polynucleotide sequence encoding at least one of an IL-2 variant, LIGHT or a variant thereof, IL-33 or a variant thereof, and CD40L or a variant thereof.

In some embodiments, the polynucleotide and the second polynucleotide are on the same vector.

In some embodiments, the cell is a lymphocyte. In some embodiments, the lymphocyte expresses: (a) the PD-1 decoy or the variant thereof and tEGFR or the variant thereof; (b) the PD-1 decoy or the variant thereof and the IL-2 variant; (c) the PD-1 decoy or the variant thereof and the LIGHT or the variant thereof; (d) the PD-1 decoy or the variant thereof and the IL-33 or the variant thereof; (e) the PD-1 decoy or the variant thereof and the CD40L or the variant thereof; (f) the PD-1 decoy or the variant thereof, the IL-2 variant, and the IL-33 or the variant thereof; (g) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the IL-2 variant; (h) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the LIGHT or the variant thereof; (i) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the IL-33 or the variant thereof; (j) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the CD40L or the variant thereof; (k) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-2 variant, and the IL-33 or the variant thereof; (l) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-2 variant, and the CD40L or the variant thereof; or (m) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-33 variant and the CD40L or the variant thereof.

In some embodiments, the lymphocyte is autologous. In some embodiments, the lymphocyte is a tumor-infiltrating lymphocyte (TIL). In some embodiments, the lymphocyte expresses a chimeric antigen receptor (CAR) or a recombinant T cell receptor (TCR). In some embodiments, the recombinant T cell receptor (TCR) shows reactivity against NY-ESO1, MAGE-A1, MAGE-A3, MAGE A-10, MAGE-C2, SSX2, MAGE-A12, or a combination thereof.

In another aspect, this disclosure additionally provides a composition comprising: (i) a polypeptide; (ii) a polynucleotide as described above; (iii) a vector; or (iv) the cell, as described above. Also within the scope of this disclosure is a pharmaceutical composition comprising an effective amount of a composition described above and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition further comprises a second therapeutic agent.

In another aspect, this disclosure additionally provides a kit comprising an effective amount of a composition or a pharmaceutical composition, as described above.

In another aspect, this disclosure further provides a method of treating a cancer/tumor or chronic infection in a subject. The method comprises administering to a subject in need thereof a therapeutically effective amount of a composition or a pharmaceutical composition, as described above.

5 In some embodiments, the cancer is selected from melanoma, sarcoma, ovarian cancer, prostate cancer, lung cancer, bladder cancer, MSI-high tumors, head and neck tumors, kidney cancer, and breast cancer.

In some embodiments, the method further comprises administering to the subject a second therapeutic agent or therapy. In some embodiments, the second therapeutic agent comprises an
10 anti-cancer or anti-tumor agent. In some embodiments, the composition or the pharmaceutical composition is administered to the subject before, after, or concurrently with the second therapeutic agent or therapy.

The foregoing summary is not intended to define every aspect of the disclosure, and additional aspects are described in other sections, such as the following detailed description. The
15 entire document is intended to be related as a unified disclosure, and it should be understood that all combinations of features described herein are contemplated, even if the combination of features are not found together in the same sentence, or paragraph, or section of this document. Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific
20 examples, while indicating specific embodiments of the disclosure, are given by way of illustration only, because various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A, 1B, 1C, and 1D are a set of diagrams showing characterization of PD-1 decoy
25 variants and hCD8⁺ T cells transduced with the PD-1 decoy variants and tEGFR. FIG. 1A shows titration ELISA of soluble monomeric PD1 decoy variants (bacterial production) against plates coated with human PDL1 protein. Bound PD1 decoy molecules were detected with anti-His tag antibody. The PD-1 decoy variant 4XMUT_M70 binds 10-fold better and the variant 6XDM about 7.5-fold better than the WT PD1 decoy to PD-L1. FIG. 1B shows detection of tEGFR and
30 intracellular PD1 decoy of retrovirally transduced CD8⁺ T cells. FIG. 1C shows IFN γ production

by NY-TCR (I53F) engineered CD8⁺ T cells co-expression PD-1 decoy (variants) and tEGFR. The engineered T cells were co-cultured at a 1:1 ratio with different PD-L1⁺ target tumor cells (100,000 of each cell type) for 48 hours. NA8 and HLA/A2⁺ NY-ESO-1⁻, SAOS2, and A375 are HLA/A2⁺ NY-ESO-1⁺. The supernatants were collected after 48 hours, diluted 1 in 25, and evaluated for the presence of IFN γ using a commercial ELISA kit (Thermo). Shown are data for a representative T-cell donor. In all assays, the variants do better than the WT PD-1 decoy. FIG. 1D shows the results of an ADCC assay of human T cells transduced to express the PD1 decoy (4XMUT_M70E) and tEGFR. CD8 T cells engineered with PD1 decoy_tEGFR retrovirus were labeled with chromium. The engineered T cells were co-cultured with anti-EGFR Ab and co-cultured with different ratios of PBMCs from the same donor. The negative control is NT (non-transduced) T cells Killing evaluated at 4 hours. As a positive control, T cells were treated with HCl.

FIGS. 2A, 2B, 2C, and 2D are a set of diagrams showing the results of an antibody-dependent cytotoxicity (ADCC) assay wherein T cells were engineered to express tEGFR. FIG. 2A shows that tEGFR engineered CD8⁺ T cells were loaded with chromium and cultured for 4-5 hours with PBMCs at different ratios along with decreasing concentrations of the anti-EGFR Ab Cetuximab. As a negative control, tCD30 engineered T cells were used in the assay along with the maximum concentration of Cetuximab (100ug/ml). Released chromium is used as a measure of lysed T cells. FIGS. 2B and 2C show the results of an ADCC assay for tHER2 engineered CD8⁺ T cells (left: Herceptin (FIG. 2B); right: Kadcyla (FIG. 2C)). FIGS. 2D shows the results of an ADCC assay for CD20 engineered CD8⁺ T cells.

FIGS. 3A, 3B, and 3C are a set of diagrams showing the representative constructs carrying transgenes used to transduce lymphocytes. The representative constructs carry a PD-1 decoy and a tEGFR (FIG. 3A), a CD40L variant (FIG. 3B), and an IL-2 variant (also referred to as IL-2^V or mutIL2) (FIG. 3C), respectively.

FIG. 4 is a diagram showing the residue numbering of WT PD-1 decoy (SEQ ID NO: 6) and other PD-1 decoy variants described in this disclosure.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure relates to novel programmed cell death 1 (PD-1) decoy variants, compositions, and methods to confer and/or increase immune responses mediated by cellular

immunotherapy, such as by adoptively transferring tumor-specific genetically-modified subsets of lymphocytes. The disclosure further provides compositions comprising genetically-modified lymphocytes that express at least two transgene(s) having the ability to modulate the immune system and the innate and adaptive immune response.

5 **A. PD-1 DECOY VARIANTS AND USE THEREOF**

10 In one aspect, this disclosure provides a PD-1 decoy polypeptide. The PD-1 decoy polypeptide comprises an amino acid sequence of a PD-1 variant, wherein the amino acid sequence has at least 80% identity to SEQ ID NO: 6 and comprises substitutions at least at positions T76, K78, and Q133 of SEQ ID NO: 6. In some embodiments, the substitutions comprise additional substitutions at least at one of positions L122 and A132. The residue numbering is shown in FIG. 4.

15 In some embodiments, the substitutions comprise: (i) T76D or a conservative substitution of D76; or T76V or a conservative substitution V76; (ii) K78R or a conservative substitution of R78; or (iii) Q133K or a conservative substitution of K133.

20 In some embodiments, the additional substitutions comprise at least one of: (i) L122E or a conservative substitution of E122; L122F or a conservative substitution of F122; or L122I or a conservative substitution of I122; and (ii) A132W or a conservative substitution of W132; A132L or a conservative substitution of L132; or A132Y or a conservative substitution of Y132.

25 In some embodiments, the amino acid sequence comprises the substitutions at the positions at T76, K78, E122, A132, and Q133. In some embodiments, the substitutions comprise: (a) T76D, K78R, L122F, and Q133K; or (b) T76V, K78R, L122F, A132W, and Q133K.

30 In some embodiments, the PD-1 decoy polypeptide further comprises a fusion partner. In some embodiments, the fusion partner comprises a fragment of a human immunoglobulin polypeptide sequence. In some embodiments, the fragment comprises: (a) a CH3 domain; and (b) part or whole of an Fc region. In some embodiments, the fragment comprises a human IgG Fc sequence (*e.g.*, IgG4Fc).

35 In some embodiments, the PD-1 decoy polypeptide further comprises a cellular elimination tag (CET). In some embodiments, the CET comprises a truncated EGFR (tEGFR) or a variant

thereof, a truncated HER2 (tHER2) or a variant thereof, a CD20 or a variant thereof, or a CD19 or a variant thereof.

In some embodiments, the fusion partner is linked (directly or indirectly via a linker) to the C-terminus of the amino acid sequence of the PD-1 variant, and the CET is linked to the C-terminus of the fusion partner.

In some embodiments, the PD-1 decoy polypeptide comprises an amino acid sequence selected from SEQ ID NOs: 9-65, 90, 92, 95-96, and 99-100.

In some embodiments, the tEGFR comprises an amino acid sequence having at least 80% (*e.g.*, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) identity to SEQ ID NO: 88 or the amino acid sequence of SEQ ID NO: 88; the HER2 comprises an amino acid sequence having at least 80% (*e.g.*, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) identity to SEQ ID NO: 93 or the amino acid sequence of SEQ ID NO: 93; and the CD20 comprises an amino acid sequence having at least 80% (*e.g.*, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) identity to SEQ ID NO: 98 or the amino acid sequence of SEQ ID NO: 98.

In some embodiments, the PD-1 decoy polypeptide comprises a detectable label.

Also within the scope of this disclosure is a polynucleotide comprising a polynucleotide sequence that encodes the PD-1 decoy polypeptide of any one of the preceding claims. In some embodiments, the polynucleotide comprises: (a) a nucleotide sequence encoding an alpha polypeptide and a beta polypeptide of a T cell receptor (TCR); or (b) a nucleotide sequence encoding a chimeric antigen receptor (CAR). Also provided is a vector comprising the polynucleotide as described above.

In another aspect, the disclosure also provides a cell comprising the described polynucleotide. In some embodiments, the cell further comprises a second polynucleotide sequence encoding at least one of an IL-2 variant, LIGHT or a variant thereof, IL-33 or a variant thereof, and CD40L or a variant thereof.

In some embodiments, the polynucleotide and the second polynucleotide are on the same vector.

As used herein, the term “variant” refers to a first molecule that is related to a second molecule (also termed a “parent” molecule). The variant molecule can be derived from, isolated

from, based on, or homologous to the parent molecule. A “functional variant” of a protein as used herein refers to a variant of such protein that retains at least partially the activity of that protein. Functional variants may include mutants (which may be insertion, deletion, or replacement mutants), including polymorphs, etc. Also included within functional variants are fusion products of such protein with another, usually unrelated, nucleic acid, protein, polypeptide, or peptide. Functional variants may be naturally occurring or may be man-made.

In some embodiments, a variant of a transgene may include one or more conservative modifications. The transgene variant with one or more conservative modifications may retain the desired functional properties, which can be tested using the functional assays known in the art.

As used herein, the term “conservative sequence modifications” refers to amino acid modifications that do not significantly affect or alter the binding characteristics of the protein containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions, and deletions. Modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include: amino acids with basic side chains (*e.g.*, lysine, arginine, histidine); acidic side chains (*e.g.*, aspartic acid, glutamic acid); uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan); nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine); beta-branched side chains (*e.g.*, threonine, valine, isoleucine); and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine) includes one or more conservative modifications. The Cas protein with one or more conservative modifications may retain the desired functional properties, which can be tested using the functional assays known in the art.

As used herein, the percent homology between two amino acid sequences is equivalent to the percent identity between the two sequences. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The

comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

The percent identity between two amino acid sequences can be determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:11-17 (1988)) which has been
5 incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970)) algorithm, which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossum62 matrix or a PAM250 matrix, and
10 a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

The term “homolog” or “homologous,” when used in reference to a polypeptide, refers to a high degree of sequence identity between two polypeptides, or to a high degree of similarity between the three-dimensional structure or to a high degree of similarity between the active site and the mechanism of action. In some embodiments, a homolog has a greater than 60% sequence
15 identity, and more preferably greater than 75% sequence identity, and still more preferably greater than 90% sequence identity, with a reference sequence. The term “substantial identity,” as applied to polypeptides, means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 75% sequence identity.

A peptide or polypeptide “fragment” as used herein refers to a less than full-length peptide, polypeptide or protein. For example, a peptide or polypeptide fragment can have at least about 3,
20 at least about 4, at least about 5, at least about 10, at least about 20, at least about 30, at least about 40 amino acids in length, or single unit lengths thereof. For example, fragment may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or more amino acids in length. There is no upper limit to the size of a peptide fragment. However, in some embodiments, peptide fragments can be less than about 500
25 amino acids, less than about 400 amino acids, less than about 300 amino acids or less than about 250 amino acids in length.

Also within the scope of this disclosure are the variants, mutants, and homologs with significant identity to the transgene. For example, such variants and homologs may have sequences with at least about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%,
30 about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%,

about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity with the sequences of transgenes described herein.

In some embodiments, the variant of the transgene as described is a fusion polypeptide comprising a transgene sequence fused (*e.g.*, N- or C-terminally fused) to a fusion partner. In some embodiments, the fusion partner comprises a fragment of a human immunoglobulin polypeptide sequence (*e.g.*, a CH3 domain; or part or whole of an Fc region, such as IgG4Fc). For example, PD-1 or a variant/fragment thereof, IL-2 or a variant/fragment thereof, IL-33 or a variant/fragment thereof, CD40L or a variant/fragment thereof, or LIGHT a variant/fragment thereof can be N- or C-terminally fused or linked, directly or indirectly via a linker, to a fusion partner, such as an IgG4Fc or a variant/fragment thereof.

The term “fusion polypeptide” or “fusion protein” means a protein created by joining two or more polypeptide sequences together. The fusion polypeptides encompassed in this invention include translation products of a chimeric gene construct that joins the nucleic acid sequences encoding a first polypeptide with the nucleic acid sequence encoding a second polypeptide to form a single open reading frame. In other words, a “fusion polypeptide” or “fusion protein” is a recombinant protein of two or more proteins which are joined by a peptide bond or via several peptides. The fusion protein may also comprise a peptide linker between the two domains.

In some embodiments, the cell is a lymphocyte. In some embodiments, the lymphocyte expresses: (a) the PD-1 decoy or the variant thereof and tEGFR or the variant thereof; (b) the PD-1 decoy or the variant thereof and the IL-2 variant; (c) the PD-1 decoy or the variant thereof and the LIGHT or the variant thereof; (d) the PD-1 decoy or the variant thereof and the IL-33 or the variant thereof; (e) the PD-1 decoy or the variant thereof and the CD40L or the variant thereof; (f) the PD-1 decoy or the variant thereof, the IL-2 variant, and the IL-33 or the variant thereof; (g) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the IL-2 variant; (h) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the LIGHT or the variant thereof; (i) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the IL-33 or the variant thereof; (j) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the CD40L or the variant thereof; (k) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-2 variant, and the IL-33 or the variant thereof; (l) the PD-1 decoy or

the variant thereof, the tEGFR or the variant thereof, the IL-2 variant, and the CD40L or the variant thereof; or (m) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-33 variant and the CD40L or the variant thereof.

In some embodiments, the lymphocyte is autologous. In some embodiments, the lymphocyte is a tumor-infiltrating lymphocyte (TIL). In some embodiments, the lymphocyte expresses a chimeric antigen receptor (CAR) or a recombinant T cell receptor (TCR). In some embodiments, the recombinant T cell receptor (TCR) shows reactivity against NY-ESO1, MAGE-A1, MAGE-A3, MAGE A-10, MAGE-C2, SSX2, MAGE-A12, or a combination thereof.

In another aspect, this disclosure additionally provides a composition comprising: (i) a polypeptide; (ii) a polynucleotide as described above; (iii) a vector; or (iv) the cell, as described above. Also within the scope of this disclosure is a pharmaceutical composition comprising an effective amount of a composition described above and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition further comprises a second therapeutic agent.

In another aspect, this disclosure additionally provides a kit comprising an effective amount of a composition or a pharmaceutical composition, as described above.

In some embodiments, lymphocytes are peripheral blood lymphocytes (PBLs). In some embodiments, lymphocytes are tumor-infiltrating lymphocytes (TILs). Lymphocytes may include T cells, B cells, NK cells, macrophages, neutrophils, dendritic cells, mast cells, eosinophils, and basophils. In some embodiments, lymphocytes are derived from CD34 hematopoietic stem cells, embryonic stem cells, or induced pluripotent stem cells. Lymphocytes can be autologous, allogeneic, syngeneic, or xenogeneic. In some embodiments, lymphocytes are autologous. In some embodiments, lymphocytes are human lymphocytes.

In some embodiments, the lymphocytes can be tumor-infiltrating lymphocytes (TILs). In some embodiments, the lymphocytes may express a chimeric antigen receptor (CAR). In some embodiments, the lymphocytes may express a recombinant T cell receptor (TCR). The CAR or TCR may bind to a cancer antigen. In some embodiments, the CAR or TCR may show reactivity against NY-ESO1, MAGE-A1, MAGE-A3, MAGE A-10, MAGE-C2, SSX2, MAGE-A12, or a combination thereof.

In some embodiments, the transgene encodes a molecule selected from a soluble receptor, a decoy, a dominant negative, a microenvironment modulator, an enzyme, an oxidoreductase, a transferase, a hydrolase, a lysase, an isomerase, a translocase, a kinase, a transporter, a modifier, a molecular chaperone, an ion channel, an antibody, a cytokine, a chemokine, a hormone, a DNA, a ribozyme, a biosensor, an epigenetic modifier, a transcriptional factor, a coding RNA, a non-coding RNA, a small-RNA, a long-RNA, an IRES element, or an exosomal-shuttle RNA.

In some embodiments, the transgene encodes at least two molecules selected from a soluble receptor, a decoy, a dominant negative, a microenvironment modulator, an enzyme, an oxidoreductase, a transferase, a hydrolase, a lysase, an isomerase, a translocase, a kinase, a transporter, a modifier, a molecular chaperone, an ion channel, an antibody, a cytokine, a chemokine, a hormone, a DNA, a ribozyme, a biosensor, an epigenetic modifier, a transcriptional factor, a coding RNA, a non-coding RNA, a small-RNA, a long-RNA, an IRES element, an exosomal-shuttle RNA, or any combination thereof.

In some embodiments, the two or more molecules encoded by the transgene are linked by a self-cleaving peptide sequence. In some embodiments, the transgene expression is regulated by a constitutively activated promoter. In some embodiments, the transgene expression is regulated by an inducible promoter. In some embodiments, the transgene expression is induced by the activation status of the lymphocyte. In some embodiments, the transgene is introduced to the lymphocytes via integration-competent gamma-retroviruses or lentivirus, DNA transposition, etc.

In some embodiments, the transgenes are selected from antibodies, antibody fragments, receptors, decoys, checkpoint blockade modulators, cytokines, chemokines, hormones, cellular elimination tags, and combinations thereof.

In some embodiments, the antibodies or antibody fragments can be VEGF, TGF- β , 4-1BB, CD28, CD27, NKG2D, PD1, PDL1, or CTLA4 antibodies. In some embodiments, the antibody is a PD1 antibody. In some embodiments, the decoy can be PD1, CTLA4, LAG3, VEGFR1, TIM3, TIGIT, or SIRPalpha decoy. In some embodiments, the decoy is a PD1 decoy, such as a PD-1.IgG4 decoy.

In some embodiments, the cytokine is selected from LIGHT or a variant/fragment thereof, IL-33 or a variant/fragment thereof, IL-2 or a variant/fragment thereof, IL-15 or a variant/fragment

thereof, IL-12 or a variant/fragment thereof, and CD40L or a variant/fragment thereof. In some embodiments, the cytokine is a mutant cytokine.

In some embodiments, the cellular elimination tag is selected from truncated tEGFR, Her2, CD20, and CD19.

5 In some embodiments, the transgenes comprise two or more of a PD-1 decoy or a variant/fragment thereof, an IL-2 variant/fragment, LIGHT or a variant/fragment thereof, IL-33 or a variant/fragment thereof, and CD40L or a variant/fragment thereof. In some embodiments, the transgenes further comprise a tEGFR or a variant/fragment thereof, a truncated HER2 (tHER2) or a variant/fragment thereof, CD20 or a variant/fragment thereof, or CD19 or a variant/fragment
10 thereof.

In some embodiments, the PD-1 decoy or a variant/fragment thereof is harbored on the same vector as a cellular elimination tag (CET), such as tEGFR or the variant/fragment thereof, tHER2 or a variant/fragment thereof, CD20 or a variant/fragment thereof, and CD19 or a variant/fragment thereof.

15 In some embodiments, the at least two transgenes comprise: (a) the PD-1 decoy or the variant/fragment thereof and tEGFR or the variant/fragment thereof; (b) the PD-1 decoy or the variant/fragment thereof and the IL-2 variant/fragment; (c) the PD-1 decoy variant thereof and the LIGHT or the variant/fragment thereof; (d) the PD-1 decoy variant thereof and the IL-33 or the variant/fragment thereof; (e) the PD-1 decoy variant thereof and the CD40L or the
20 variant/fragment thereof; (f) the PD-1 decoy variant thereof, the IL-2 variant/fragment, and the IL-33 or the variant/fragment thereof; (g) the PD-1 decoy variant thereof, the tEGFR or the variant/fragment thereof, and the IL-2 variant/fragment; (h) the PD-1 decoy variant thereof, the tEGFR or the variant/fragment thereof, and the LIGHT or the variant/fragment thereof; (i) the PD-1 decoy variant thereof, the tEGFR or the variant/fragment thereof, and the IL-33 or the
25 variant/fragment thereof; (j) the PD-1 decoy variant thereof, the tEGFR or the variant/fragment thereof, and the CD40L or the variant/fragment thereof; (k) the PD-1 decoy variant thereof, the tEGFR or the variant/fragment thereof, the IL-2 variant/fragment, and the IL-33 or the variant/fragment thereof; (l) the PD-1 decoy variant thereof, the tEGFR or the variant/fragment thereof, the IL-2 variant/fragment, and the CD40L or the variant/fragment thereof; or (m) the PD-

1 decoy variant thereof, the tEGFR or the variant/fragment thereof, the IL-33 variant/fragment and the CD40L or the variant/fragment thereof.

In some embodiments, the composition comprises at least two subsets of lymphocytes. For example, the composition may include two, three, four, five or more genetically-modified subsets of lymphocytes. Each subset of genetically-modified lymphocytes may express at least one transgene. For example, each subset of genetically-modified lymphocytes may express two, three, four, five or more transgenes.

In some embodiments, the composition comprises two genetically-modified subsets of lymphocytes, in which each subset expresses at least one transgene. In some embodiments, the composition comprises two genetically-modified subsets of lymphocytes, wherein each subset expresses two transgenes. In some embodiments, the composition comprises three genetically-modified subsets of lymphocytes, wherein each subset expresses at least one transgene. In some embodiments, the composition comprises four genetically-modified subsets of lymphocytes, wherein each subset expresses at least one transgene. In some embodiments, the composition comprises five or more genetically-modified subsets of lymphocytes, wherein each subset expresses at least one transgene.

In some embodiments, the composition comprises at least two genetically-modified subsets of lymphocytes, wherein each subset expresses at least two transgenes and wherein each subset shares one transgene. In some embodiments, the composition comprises at least two genetically-modified subsets of lymphocytes, wherein each subset expresses at least two transgenes and wherein each subset expresses different transgenes.

In some embodiments, the plurality of lymphocytes may include: (i) a first subset expressing at least two transgenes; and (ii) a second subset expressing at least two transgenes, wherein at least one of the transgenes of the first subset is different from the transgenes of the second subset or wherein at least one of the transgenes of the first subset is in common with the transgenes of the second subset. In some embodiments, the composition of lymphocytes may express three transgenes after combining the first subset and the second subset.

In some embodiments, (i) the first subset expresses at least a PD-1 decoy or a variant/fragment thereof and an IL-2 variant/fragment and the second subset expresses at least a PD-1 decoy or a variant/fragment thereof and LIGHT or a variant/fragment thereof; (ii) the first

subset expresses at least a PD-1 decoy or a variant/fragment thereof and an IL-2 variant/fragment and the second subset expresses at least a PD-1 decoy or a variant/fragment thereof and IL-33 or a variant/fragment thereof; (iii) the first subset expresses at least a PD-1 decoy or a variant/fragment thereof and an IL-2 variant/fragment and the second subset expresses at least a PD-1 decoy or a variant/fragment thereof and CD40L or a variant/fragment thereof; (iv) the first subset expresses at least a PD-1 decoy or a variant/fragment thereof and LIGHT or a variant/fragment thereof and the second subset expresses at least a PD-1 decoy or a variant/fragment thereof and IL-33 or a variant/fragment thereof; or (v) the first subset expresses at least a PD-1 decoy or a variant/fragment thereof and LIGHT or a variant/fragment thereof and the second subset expresses at least a PD-1 decoy or a variant/fragment thereof and CD40L or a variant/fragment thereof; or (vi) the first subset expresses at least a PD-1 decoy or a variant/fragment thereof and IL-33 or a variant/fragment thereof and the second subset expresses at least a PD-1 decoy or a variant/fragment thereof and CD40L or a variant/fragment thereof.

In some embodiments, the first subset or the second subset further expresses a tEGFR or a variant thereof, a truncated HER2 (tHER2) or a variant thereof, CD20 or a variant thereof, or CD19 or a variant thereof.

In some embodiments, (i) the first subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and an IL-2 variant/fragment and the second subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and LIGHT or the variant/fragment thereof; (ii) the first subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and an IL-2 variant/fragment and the second subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and IL-33 or the variant/fragment thereof; (iii) the first subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and an IL-2 variant/fragment and the second subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and CD40L or the variant/fragment thereof; (iv) the first subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and LIGHT or the variant/fragment thereof and the second subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and IL-33 or the variant/fragment thereof; (v) the first subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and LIGHT or the variant/fragment thereof and the

second subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and CD40L or the variant/fragment thereof; or (vi) the first subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and IL-33 or the variant/fragment thereof and the second subset expresses at least the PD-1 decoy or the variant thereof, tEGFR or the variant/fragment thereof and CD40L or the variant/fragment thereof.

Immunosuppressive polypeptides known to suppress or decrease an immune response via their binding include CD47, PD-1, CTLA-4, and their corresponding ligands, including SIRPalpha, PD-L1, PD-L2, B7-1, and B7-2. Such polypeptides are present in the tumor microenvironment and inhibit immune responses to neoplastic cells. In various embodiments, inhibiting, blocking, or antagonizing the interaction of immunosuppressive polypeptides and/or their ligands via a transgene enhances the immune response of the immunoresponsive cell. In one aspect, a transgene can function as a gene knock-down for inhibitory/checkpoint molecules, including, but not limited to, PD-1, CTLA-4, LAG-3, TIGIT, VISTA, TIM-3, and CBL-B.

Co-stimulatory polypeptides known to stimulate or increase an immune response via their binding include CD28, OX-40, 4-1BB, CD27, and NKG2D and their corresponding ligands, including B7-1, B7-2, OX-40L, 4-1BBL, CD70, and NKG2D ligands. Such polypeptides are present in the tumor microenvironment and activate immune responses to neoplastic cells. In various embodiments, promoting, stimulating, or agonizing pro-inflammatory polypeptides and/or their ligands via a transgene enhances the immune response of the immunoresponsive cell.

In some embodiments, transgenes are cytokines or growth factors. The terms “growth factors” and “cytokines” mean signaling molecules that control cell activities in an autocrine, paracrine or endocrine manner. They exert their biological functions by binding to specific receptors and activating associated downstream signaling pathways, which, in turn, regulate gene transcription in the nucleus and ultimately stimulate a biological response (Nicola N. Oxford; New York: Oxford University Press; 1994). Growth factors and cytokines affect a wide variety of physiological processes such as cell proliferation, differentiation, apoptosis, immunological or hematopoietic response, morphogenesis, angiogenesis, metabolism, wound healing, and maintaining tissue homeostasis in adult organisms. Historically, growth factors were thought to be biological moieties that have a positive effect on cell growth and proliferation, while cytokines

were typically considered to have an immunological or hematopoietic response. However, as different lines of research have converged, it has been found that “cytokines” and “growth factors” can have similar functions, and therefore, these terms are herein used interchangeably.

TGF- β Superfamily: The TGF-beta superfamily includes the TGF-beta proteins, Bone Morphogenetic Proteins (BMPs), Growth Differentiation Factors (GDFs), Glial-derived Neurotrophic Factors (GDNFs), Activins, Inhibins, Nodal, Lefty, and Müllerian Inhibiting Substance (MIS). The TGF-beta superfamily members are multifunctional regulators of various biological processes such as morphogenesis, embryonic development, adult stem cell differentiation, immune regulation, wound healing, inflammation, and cancer.

(1) BMP-like family: (BMPs (*i.e.*, BMP1-10, BMP-15), GDFs (*i.e.*, GDF1-15), AMH

(2) GDNFs Family: GDNF, Artemin, Neurturin, and Persephone

(3) TGF- β -like Family: TGF- β s (*i.e.*, TGF- β -1, TGF- β -2, TGF- β -3), Activins (*i.e.*, Activin A/AB/B, Inhibin A/B), Nodal

Epidermal Growth (EGFs) Factors: The EGF family members include EGF, TGF- α , Neuregulins, Amphiregulin, Betacellulin, and others. The members of the EGF family are best known for their ability to stimulate cell proliferation, differentiation, and survival. Deregulation of the members of this family and their receptors is closely associated with tumorigenesis (Herbst RS. International Journal of Radiation Oncology, Biology, Physics 2004, 59(2 Suppl):21-26).

Platelet-Derived Growth Factors (PDGFs): Platelet-derived growth factors (PDGFs) are potent mitogenic and chemotactic proteins. There are currently four known PDGF proteins encoded by four genes (PDGFA, PDGFB, PDGFC, and PDGFD). PDGFs are secreted as disulfide-linked homodimers or heterodimers that include PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, and PDGF-AB. There are two known PDGF receptors with intrinsic tyrosine kinase activity; PDGFR α and PDGFR β , both of which can form heterodimers and homodimers. Ligand binding promotes receptor dimerization, autophosphorylation, and the consequent activation of multiple downstream intracellular signaling cascades. Signaling via PDGFR α is essential for the development of the facial skeleton, hair follicles, spermatogenesis oligodendrocytes and astrocytes, as well as for the development of the lung and intestinal villi while signaling via

PDGFR β is crucial for the development of blood vessels, kidneys and white adipocytes (Heldin CH. Cell Commun Signal 2013, 11:97).

Fibroblast Growth Factors (FGFs) Family: In humans, twenty-two members of the FGF family have been identified, all of which are heparin-binding proteins. High-affinity interactions with cell-surface-associated heparan sulfate proteoglycans are essential for FGF signal transduction as mediated by receptor tyrosine kinases (Ornitz DM, Itoh N. Genome Biology 2001, 2(3): REVIEWS3005). FGFs are pluripotent proteins that are primarily mitogenic but also have regulatory, morphological, and endocrine effects. FGFs are involved in embryonic developmental processes (Heldin CH: Targeting the PDGF signaling pathway in tumor treatment. Cell Commun Signal 2013, 11:97), mature tissues/systems angiogenesis (Kim BS, *et al.* Biochemical and biophysical research communications 2014, 450(4):1333-1338), keratinocyte organization (Tsuboi R, *et al.* The Journal of Investigative Dermatology 1993, 101(1):49-53) and wound healing processes (Lee JG, Kay EP. Investigative Ophthalmology & Visual Science 2006, 47(4):1376-1386).

Insulin-like Growth Factors (IGFs): The Insulin-like Growth Factors (IGFs) are proteins with high sequence similarity to Insulin. The IGF receptor is a disulfide-linked heterotetrameric transmembrane protein with a cytoplasmic tyrosine kinase domain. There are two types of IGF receptors, IGFI-R and IGFII-R. The availability of IGFs can be regulated by IGF Binding Proteins 1-6 (Griffeth RJ, *et al.* Basic and clinical andrology 2014, 24:12). The primary action of IGFs is on cell growth. Indeed, most of the actions of pituitary growth hormone are mediated by IGFs, primarily IGF-1. Growth hormone stimulates many tissues, particularly the liver, to synthesize and secrete IGF-1, which in turn stimulates both hypertrophy (increase in cell size) and hyperplasia (increase in cell number) in most tissues, including bone. IGFs can also induce neuron survival, protect cartilage cells, and activate osteocytes (Brahmkhatri VP, *et al.* BioMed research international 2015, 2015:538019).

Vascular Endothelial Growth Factors (VEGFs): VEGFs are homodimeric, glycoprotein growth factors that are specific to endothelial cells (Ferrara N, Gerber HP, LeCouter. Nature Medicine 2003, 9(6):669-676). They regulate angiogenesis and vascular permeability, especially during embryogenesis, skeleton growth, and reproductive functions. They also play important roles in hematopoiesis. VEGFs signal mainly through tyrosine kinases VEGFR1 and VEGFR2 and

stimulate cell survival, proliferation, migration, and/or adhesion (Ferrara N. Endocrine Reviews 2004, 25(4):581-611). Deregulation of VEGFs has been associated with tumors, intraocular neovascular disorders, and other diseases (Ferrara N, *et al.* Nature Medicine 2003, 9(6):669-676). Members of the VEGF gene family include VEGF/VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and Placental Growth Factor (PlGF) (Holmes DI, Zachary I. Genome Biology 2005, 6(2):209).

Hepatocyte Growth Factors (HGFs): HGF is secreted by mesenchymal cells and acts as a multi-functional cytokine on cells that are mainly of epithelial and endothelial origin. It regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade via HGFR (Okada M, *et al.* Pediatric Research 2004, 56(3):336-344). HGF has been shown to have a major role in embryonic organ development, adult organ regeneration, and wound healing. Furthermore, its ability to stimulate mitogenesis, cell motility, and matrix invasion gives it a central role in angiogenesis and tumorigenesis (Sharma NS, *et al.* FASEB 2010, 24(7):2364-2374).

Tumor necrosis factors (TNFs): Cytokines that were known to be involved in tumor cell apoptosis were initially classified as Tumor Necrosis Factors (or under the TNF family). All TNF family members share a trimeric, conserved C-terminal domain called the 'TNF homology domain' or THD. Responsible for receptor binding, THD shares a ~20–30% sequence identity amongst family members. Although most ligands are synthesized as membrane-bound proteins, soluble forms can be generated by limited proteolysis (Bodmer JL, *et al.* Trends in Biochemical Sciences 2002, 27(1):19-26). The first two members of the family to be identified were TNF α and TNF β . To date, 19 TNF superfamily ligands have been identified along with 32 TNF superfamily receptors. While many TNF superfamily members promote or inhibit apoptosis, they also regulate critical functions of both the innate and adaptive immune system, including natural killer cell activation, T-cell co-stimulation, and B-cell homeostasis and activation (Croft M. Nature Reviews Immunology 2009, 9(4):271-285). LIGHT (homologous to lymphotoxin, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) is a type II transmembrane glycoprotein of the TNF ligand superfamily. LIGHT is expressed on immature DCs and activated T cells and binds to 3 distinct receptors, herpes virus entry mediator (HVEM), lymphotoxin- β receptor (LT β R), and decoy receptor 3/TR6. Upon binding to HVEM, LIGHT costimulates T cells and accelerates proliferation

and cytokine production. Another example is CD154, also called CD40 ligand or CD40L. It is a protein that is primarily expressed on activated T cells and is a member of the TNF superfamily of molecules. It binds to CD40 on antigen-presenting cells, which leads to many effects depending on the target cell type. Yet another example is Fas ligand (FasL or CD95L or CD178). Fas ligand/receptor interactions play an important role in the regulation of the immune system and the progression of cancer.

Interleukins (ILs): Interleukins are a large group of immunomodulatory proteins that regulate growth, differentiation, and activation of cells in the immune or hematopoietic systems during the immune response. Based on distinguishing structural features, the known ILs can be divided into four major groups that include; the IL1-like cytokines, the class I helical cytokines (IL4-like, γ -chain, and IL-6/12-like), the class II helical cytokines (IL-10-like and IL-28-like), and the IL-17-like cytokines (Table 1).

IL4-like	IL-3, IL-4, IL-5, IL-13, CSF2	Four tightly packed α -helices known as “four-helix bundle” motif; shorter core helices
IL--6/12-like	IL-6, IL-11, IL-12A, IL-23A, IL-27A, IL-31, CLCF1, CNTF, CTF1, LIF, OSM, CSF3	Four tightly packed α -helices known as “four-helix bundle” motif; longer core helices
IL10-like	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26	“Bundle helix” structural motif-containing six or seven stacked helices
IL-28-like	IL-28A, IL-28B, IL-29	“Bundle helix” structural motif-containing six or seven stacked helices
IL-17-like	IL-17A, IL-17B, IL-17C, IL-17D, IL-25, IL-17F	Neurotrophin-like cysteine-knot fold
Non-classified	IL-8, TXLNA, IL-16, IL-32, IL-34, CSF1	Varies

Interferons (IFNs): IFNs are a group of signaling proteins that are made and released by host cells in response to the presence of pathogens such as viruses, bacteria, parasites, or tumor cells. Interferons also have immunoregulatory functions; they inhibit B-cell activation, enhance T-cell activity, and increase the cellular-destruction capability of natural killer cells. More than twenty distinct IFN genes and proteins have been identified in animals, including humans. They are typically divided into two classes: Type I IFN and Type II IFN. Type I IFNs are also known

as viral IFNs and include IFN- α , IFN- β , and IFN- ω . Type II IFN is also known as immune IFN (IFN- γ). The viral IFNs are induced by virus infection, whereas type II IFN is induced by mitogenic or antigenic stimuli. Most types of virally infected cells are capable of synthesizing Type I IFN in cell culture. By contrast, IFN- γ is synthesized only by certain cells of the immune system, including natural killer cells, CD4 Th1 cells, and CD8 cytotoxic suppressor cells (Samuel CE. Clinical Microbiology Reviews 2001, 14(4):778-809, table of contents).

In some embodiments, a transgene is a decoy receptor. A “decoy receptor” means a receptor that is able to recognize and bind specific growth factors or cytokines efficiently, but is not structurally able to signal or activate the intended receptor complex. It acts as an inhibitor, binding a ligand and keeping it from binding to its regular receptor.

In some embodiments, a transgene is a soluble decoy. A “soluble decoy” means a polypeptide that is expressed and secreted from a cell and that binds to a specific receptor on a different cell, therefore, inhibiting the binding of its native ligand to such receptor. Non-limiting examples of soluble decoys are PD1-decoy, CTLA-4 decoy, LAG3-decoy, VEGFR1 decoy, TIM3 decoy, TIGIT decoy, and SIRPalpha decoy. In one embodiment, PD-1 decoys are expressed and secreted by lymphoid cells, and such PD-1 decoys inhibit binding of native PD-1 on T-cells to PDL-1 on antigen-presenting cells (APCs) by occupying the binding site of PD-L1 on APCs thus inhibiting immunosuppressive signaling of T-cells and therefore enhancing the immune response of the T-cells.

PD-1 decoy: PD-1 is a strong negative regulator of T lymphocytes in the tumor microenvironment. In one embodiment, T cells were generated expressing a dominant-negative deletion mutant of PD-1 (a non-limiting example of a PD-1 decoy) via retroviral transduction. This PD-1 decoy increased IFN- γ secretion of antigen-specific T cells in response to tumor cells expressing the cognate antigen. In another embodiment, soluble fragments of the PD-1 ectodomain (a non-limiting example of a PD-1 decoy) that have higher binding affinity to PDL-1 are administered as competitive antagonists of PDL-1. Non-limiting examples of soluble PD-1 ectodomain variants are disclosed in Maute *et al.* PNAS 2015 Nov 24; 112(47): E6506-E6514. In yet another embodiment, a PD-1 decoy molecule comprising the ectodomain of PD1 fused to the Fc region of human IgG4 (PD-1.IgG4) can be used for enhanced tumor control *in vivo*. In another embodiment, such a PD-1 decoy can be expressed and secreted by TILs.

The PD-1 decoy, as described in this disclosure, can also be generated by computational-based rational design to develop binding and/or solubility enhanced variants of the ectodomain of PD-1. For example, single and multiple amino acid replacements predicted to increase the binding affinity of PD-1 for PD-L1 are evaluated in a recombinant soluble protein produced in a bacterial expression system. The variants can be evaluated by direct titration ELISA for binding to plate-captured PD-L1. Variants of interest can then be cloned into retroviral vectors for evaluation of secretion by T cells. PD-1 decoys that demonstrate poor solubility during bacterial production are discarded because typically poor solubility corresponds to no or low production by T cells.

PD-1 decoys produced by engineered human T cells may also comprise an Fc portion (*e.g.*, IgG4Fc) to increase avidity and stability of the protein. PD1-Fc decoys produced by primary human T cells can be evaluated in ELISA. To evaluate functionality, a co-culture assay was established in which primary human T cells co-engineered to express the A2/NY-ESO-1 T cell receptor (TCR) to allow tumor cell recognition (by lentivirus transduction) as well as the PD1-Fc decoy and the cell-surface tEGFR (encoded in a bicistronic retroviral vector). These co-transduced T cells (or control T cells comprising TCR only or PD1 decoy only) were co-cultured with target tumor cells that are PDL1^{pos}. IFN γ levels present in the co-culture supernatant were evaluated to determine the best PD-1 decoy variant (*i.e.*, the higher the IFN γ level, the better the PD1 decoy at blocking PD-L1 on the target tumor cell surface). 4XMUT_M70 and 6XDM are among the PD1-Fc decoy variants showing high binding affinity to PD-L1 and high solubility (FIGS. 1A-D).

Cellular Elimination Tag (CET): The transgenes, such as PD1-Fc decoy, can be expressed constitutively from a bicistronic retroviral vector also encoding a CET, such as tEGFR, tHER2, CD20, or CD19 (FIGS. 2A-D). The purpose of the CET is four-fold. First of all, it can be used as a means of evaluating transduction efficiency and second for enriching the engineered cells (on anti-EGFR coated beads) if necessary. Third, it can be used as a means of tracking the engineered T cells in a patient post-engraftment (via FACS from drawn blood samples or tumor biopsies). And finally, it can be used as an elimination tag via ADCC in the event of toxicity in a patient with Cetuximab. A truncated human EGFR polypeptide (huEGFRt) that is devoid of extracellular N-terminal ligand binding domains and intracellular receptor tyrosine kinase activity but retains the native amino acid sequence, type I transmembrane cell surface localization, and a conformationally intact binding epitope for pharmaceutical-grade anti-EGFR monoclonal antibody, cetuximab (Erbix) is described in Wang *et al.* (Wang X, *et al.* Blood. 2011 Aug

4;118(5):1255-63. Epub 2011 Jun 7). Other examples of CETs ADCC may include tHER2 (with Herceptin or Kadcyla), CD20 (with Rituximab), and CD19. CD20 as a CET is described in Griffioen *et al.* (Griffioen M, *et al.* Haematologica. 2009 Sep;94(9):1316-20). CD19 as a CET is described in Budde *et al.* (Budde, *et al.* Blood 2013; 122 (21): 1660) and Annesley *et al.* (Annesley *et al.*, Blood 2019; 134 (Supplement_1): 223).

LIGHT: LIGHT is a type II transmembrane glycoprotein of the TNF ligand superfamily (Mauri *et al.* Immunity 1998 Jan; 8(1):21-30). It is expressed on immature dendritic cells and activated T cells (Tamada K *et al.* J Immunol. 2000 Apr 15; 164(8):4105-10) and binds to 3 distinct receptors, herpes virus entry mediator (HVEM), lymphotoxin- β receptor (LT β R), and decoy receptor 3/TR6. Upon binding to HVEM, LIGHT costimulates T cells and accelerates proliferation and cytokine production (Tamada *et al.* Nat Med. 2000 Mar; 6(3):283-9). In one embodiment, LIGHT protein can be engineered to express and secreted from TILs.

IL-33: Cytokines are central mediators between cells in the inflammatory tumor microenvironment, in which Interleukin-33 (IL-33) is considered as an alarmin released after cellular damage. IL-33 was discovered as a member of the IL-1 family of cytokines. The IL-1 gene family contains 11 members (IL-1 α , IL-1 β , IL-1RA, IL-18, IL-36RA, IL-36 α , IL-37, IL-36 β , IL-36 γ , IL-38, IL-33), which induces a complex network of pro-inflammatory cytokines and regulates and initiates inflammatory responses, via expressing integrins on leukocytes and endothelial cells (Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. (Dinarello CA., Blood. 2011 Apr 7; 117(14):3720-32). The process of tumor development can trigger anti-tumor immune responses. The type 1 immune response is a critical component of cell-mediated immunity, which includes tumor-induced IFN- γ -producing Th1 cells, cytotoxic T lymphocytes, NK T cells, and $\gamma\delta$ T cells, to limit tumor growth and metastasis (Galon *et al.* Science. 2006 Sep 29; 313(5795):1960-4.). Since inflammation is another important component in malignancies, IL-33 can play roles in improving cancerous surveillance and immunity against tumors. In one embodiment of the present invention, IL-33 can be engineered to express and secreted from TILs.

IL-2: Interleukin-2 (IL-2) was one of the first cytokines discovered to be molecularly characterized. It was primarily shown to support the growth and expansion of T and NK cells. IL-2 was approved for clinical use in 1992, but the precise description of the biology of its receptor is still under study. Systemic high dose (HD) IL-2 treatment produces durable responses in

melanoma and renal cancer carcinoma patients, but only in a relatively small fraction of patients. Moreover, systemic HD IL-2 treatments induce significant toxicities, further limiting its clinical relevance. IL-2 promotes the activation and expansion of T cells and NK cells *in vitro*. In one embodiment, IL-2 or its functional variants can be engineered to express and secreted from TILs.

Such TILs can further be engineered to secrete additional transgenes.

CD40L: As immune co-stimulatory molecules, CD40 and its ligand CD40L can complement each other. Previous studies have shown that CD40 and CD40L play pivotal roles in humoral and cellular immunity, and the expression of CD40 and CD40L are closely related to the occurrence and development of various diseases (Elgueta *et al.* Immunol Rev 2009; 229:152-172). CD40 was found to be highly expressed in bladder cancer, breast cancer, ovarian cancer, and other tumors (Hussain *et al.* Br J Cancer 2011; 88:586). CD40L, as the primary ligand of CD40, is mainly expressed on the surface of activated CD4⁺ T cells. When CD40 binds CD40L, CD40L can activate T lymphocytes and the Fas-mediated apoptotic pathway in tumor cells.

In another aspect, the above-described genetically-modified lymphocytes can be incorporated into pharmaceutical compositions suitable for administration. The pharmaceutical compositions generally comprise substantially isolated/purified lymphocytes and a pharmaceutically acceptable carrier in a form suitable for administration to a subject. Pharmaceutically-acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. The pharmaceutical compositions are generally formulated in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

The terms “pharmaceutically acceptable,” “physiologically tolerable,” as referred to compositions, carriers, diluents, and reagents, are used interchangeably and include materials are capable of administration to or upon a subject without the production of undesirable physiological effects to the degree that would prohibit administration of the composition. For example, “pharmaceutically-acceptable excipient” includes an excipient that is useful in preparing a pharmaceutical composition that is generally safe, non-toxic, and desirable, and includes excipients that are acceptable for veterinary use as well as for human pharmaceutical use.

Examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. The use of such media and compounds

for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or compound is incompatible with the disclosed composition, use thereof in the compositions is contemplated. In some embodiments, a second therapeutic agent, such as an anti-cancer or anti-tumor, can also be incorporated into pharmaceutical compositions.

5 Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water-soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate-buffered saline (PBS). In all cases, the composition must be sterile and should be fluid
10 to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, *e.g.*, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, *e.g.*, by the use of a coating such
15 as lecithin, by the maintenance of the required particle size in the case of dispersion, and by the use of surfactants.

In some embodiments, the composition includes the genetically-modified lymphocytes as described above and optionally a cryo-protectant (*e.g.*, glycerol, DMSO, PEG).

The composition or the pharmaceutical composition described herein can be provided in a
20 kit. In one embodiment, the kit includes (a) a container that contains the composition and optionally (b) informational material. The informational material can be descriptive, instructional, marketing or other material that relates to the methods described herein and/or the use of the agents for therapeutic benefit. For example, kits may include instruction for the manufacturing, for the therapeutic regimen to be used, and periods of administration. In an embodiment, the kit includes
25 also includes an additional therapeutic agent (*e.g.*, a checkpoint modulator). The kit may comprise one or more containers, each with a different reagent. For example, the kit includes a first container that contains the composition and a second container for the additional therapeutic agent.

The containers can include a unit dosage of the pharmaceutical composition. In addition to the composition, the kit can include other ingredients, such as a solvent or buffer, an adjuvant,
30 a stabilizer, or a preservative.

The kit optionally includes a device suitable for administration of the composition, *e.g.*, a syringe or other suitable delivery device. The device can be provided pre-loaded with one or both of the agents or can be empty, but suitable for loading.

B. METHODS FOR PREPARING THE COMPOSITIONS

5 In another aspect, this disclosure further provides a method of preparing the above-described composition. The method comprises: (a) providing a plurality of lymphocytes; (b) introducing to the plurality of lymphocytes a nucleic acid molecule encoding at least two transgenes to obtain a plurality of genetically-modified lymphocytes; and (c) expanding the plurality of genetically-modified in a cell culture medium.

10 In some embodiments, the method may include: (a) providing a plurality of lymphocytes; (b) introducing to the plurality of lymphocytes two or more nucleic acid molecules, each of the two or more nucleic acid molecules encoding at least one transgene, thereby obtaining a plurality of genetically-modified lymphocytes; and (c) expanding the plurality of genetically-modified in a cell culture medium.

15 In some embodiments, the transgenes comprise two or more of a PD-1 decoy, an IL-2 variant/fragment, LIGHT or a variant/fragment thereof, IL-33 or a variant/fragment thereof, and CD40L or a variant/fragment thereof. In some embodiments, the transgenes further comprise a truncated EGFR (tEGFR) or a variant/fragment thereof. In some embodiments, the PD-1 decoy or the variant thereof and the tEGFR or the variant/fragment thereof (or the truncated HER2 (tHER2)
20 or a variant/fragment thereof, CD20 or a variant/fragment thereof, or CD19 or a variant/fragment thereof) are harbored on the same vector.

In some embodiments, the at least two transgenes comprise: (a) the PD-1 decoy or the variant thereof and tEGFR or the variant thereof; (b) the PD-1 decoy or the variant thereof and the IL-2 variant; (c) the PD-1 decoy or the variant thereof and the LIGHT or the variant thereof; (d)
25 the PD-1 decoy or the variant thereof and the IL-33 or the variant thereof; (e) the PD-1 decoy or the variant thereof and the CD40L or the variant thereof; (f) the PD-1 decoy or the variant thereof, the IL-2 variant, and the IL-33 or the variant thereof; (g) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the IL-2 variant; (h) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the LIGHT or the variant thereof; (i) the PD-1 decoy or the
30 variant thereof, the tEGFR or the variant thereof, and the IL-33 or the variant thereof; (j) the PD-

1 decoy or the variant thereof, the tEGFR or the variant thereof, and the CD40L or the variant thereof; (k) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-2 variant, and the IL-33 or the variant thereof; (l) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-2 variant, and the CD40L or the variant thereof; or (m) the PD-1
5 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-33 variant and the CD40L or the variant thereof.

In some embodiments, the method may include: (a) introducing to a first plurality of lymphocytes a first nucleic acid molecule encoding at least two transgenes to obtain a first plurality of genetically-engineered lymphocytes; and (b) introducing to a second plurality of lymphocytes
10 a second nucleic acid molecule encoding at least two transgenes to obtain a second plurality of genetically-engineered lymphocytes. In some embodiments, the method further comprises combining the first plurality of genetically-engineered lymphocytes with the first plurality of genetically-engineered lymphocytes at a predetermined ratio between about 1:1 and about 1:100 (*e.g.*, 1:1, 1:2, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 1:60, 1:70, 1:80, 1:90, 1:100).

15 In some embodiments, the method includes: a) introducing transgenes in different lymphocytes subsets, wherein each subset expresses at least one transgene, and b) combining at least two subsets of lymphocytes. In some embodiments, each subset expresses at least two transgenes according to the embodiments described above. In some embodiments, the composition of lymphocytes expresses at least three different transgenes.

20 In some embodiments, methods to obtain a composition of tumor-specific genetically-modified subsets of lymphocytes described above can be performed *in vitro* or *ex vivo*. Methods in more particular form may be as disclosed in PCT/EP2018/080343, the content of which is hereby incorporated by reference in its entirety.

In some embodiments, the method may additionally include expanding the first plurality
25 of lymphocytes in a cell culture medium following the step of introducing the first nucleic acid or expanding the second plurality of lymphocytes in a cell culture medium following the step of introducing the second nucleic acid.

The term “culturing” or “expanding” refers to maintaining or cultivating cells under conditions in which they can proliferate and avoid senescence. For example, cells may be cultured
30 in media optionally containing one or more growth factors, *i.e.*, a growth factor cocktail. In some

embodiments, the cell culture medium is a defined cell culture medium. The cell culture medium may include neoantigen peptides. Stable cell lines may be established to allow for the continued propagation of cells.

a. Lymphocytes

Prior to the expansion and genetic modification of the lymphocytes described herein, a source of lymphocytes from a subject is obtained. Lymphocytes can be obtained from several sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, umbilical cord blood, thymus tissue, tissue from an infection site, ascites, pleural effusion, splenic tissue, and tumors. As described herein, any number of lymphocyte lines available in the art can be used.

Lymphocytes can be obtained from a unit of blood collected from a subject using any number of techniques known to the person skilled in the art, such as the Ficoll™ separation. Circulating blood cells of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T lymphocytes, monocytes, granulocytes, B lymphocytes, other nucleated white blood cells, red blood cells, and platelets. The cells harvested by apheresis can be washed to remove the plasma fraction and place the cells in a suitable buffer or medium for the subsequent processing steps. The cells may be washed with phosphate-buffered saline (PBS). Alternatively, the wash solution may lack calcium and may lack magnesium or may lack many, if not all, divalent cations. As those of ordinary skill in the art would readily appreciate, a washing step can be achieved by methods known to those skilled in the art, such as using a semiautomatic continuous flow centrifuge (*e.g.*, the Cobe 2991 cell processor, the Baxter CytoMate, or elHaemonetics Cell Saver 5) according to the manufacturer's instructions. After washing, the cells can be resuspended in a variety of biocompatible buffers, such as, for example, Ca²⁺ free, PBS free Mg²⁺, PlasmaLyte A, or other saline solution with or without buffer. Alternatively, the undesirable components of the apheresis sample can be removed and the cells resuspended directly in a culture medium.

As described herein, lymphocytes may be isolated from peripheral blood by lysis of red blood cells and depletion of monocytes, for example, by centrifugation through a PERCOLL™ gradient or by countercurrent centrifugal elutriation. If needed, specific subpopulation lymphocytes, such as T lymphocytes (*i.e.*, Cd3⁺, CD28⁺, CD4⁺, CD8⁺, CD45RA⁺ or CD45RO⁺ T lymphocytes) can be further isolated by positive or negative selection techniques. For example, T lymphocytes may be isolated by incubation with conjugated anti-CD3 / anti-CD28 beads (*i.e.*,

3x28), such as DYNABEADS® M-450 CD3 / CD28 T, for a sufficient period of time (*i.e.*, 30 minutes to 24 hours) for positive selection of the desired T lymphocytes. For the isolation of T lymphocytes from patients with leukemia, the use of longer incubation times, such as 24 hours, can increase cellular performance. Longer incubation times can be used to isolate T lymphocytes in any situation where there are few T lymphocytes compared to other cell types, such as isolating tumor-infiltrating lymphocytes (TILs) from tumor tissue or from immunocompromised individuals. The person skilled in the art will recognize that multiple rounds of selection may also be used. It may be desirable to perform the selection procedure and use the “unselected” cells in the activation and expansion process. “Unselected” cells can also undergo new rounds of selection.

Enrichment of a population of lymphocytes (*e.g.*, T lymphocytes) by negative selection can be performed with a combination of antibodies directed to unique surface markers for the negatively selected cells. One method is the sorting and/or selection of cells by negative magnetic immune adherence or flow cytometry using a cocktail of monoclonal antibodies directed to cell surface markers present in the negatively selected cells. For example, to enrich CD4⁺ cells by negative selection, a monoclonal antibody typically includes antibodies against CD14, CD20, CD11b, CD16, HLA-DR, and CD8. Alternatively, the regulatory T lymphocytes are depleted by anti-C25 conjugate beads or other similar selection method.

Lymphocytes for stimulation can also be frozen after a washing step. Wishing not to be bound by theory, freezing and the following thawing step provide a more uniform product by eliminating granulocytes and, to some extent, monocytes in the cell population. After the washing step that removes the plasma and platelets, the cells can be suspended in a freezing solution. Although many solutions and freezing parameters are known in the art and will be useful in this context, one method involves the use of PBS containing 20% DMSO and 8% human serum albumin, or culture medium containing 10% dextran 40 and 5% dextrose human albumin and 7.5% DMSO or 31.25% Plasmalyte A, 31.25% dextrose 5%, 0.45% NaCl, 10% dextran 40 and 5% of dextrose, 20% serum of human albumin and 7.5% of DMSO or other suitable cell freezing medium containing, for example, Hespan and PlasmaLyte A. The cells may then be frozen at -80°C at a rate of 1°C per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing can be used, as well as uncontrolled freezing immediately at -20°C or in liquid nitrogen.

The cryopreserved cells may be thawed and washed as described herein and allowed to stand for one hour at room temperature before activation using the methods of the present invention. As described herein, lymphocytes can be expanded, frozen, and used later. As described herein, samples may be collected from a patient shortly after the diagnosis of a particular disease as described herein, but before any treatment. The cells may be isolated from a blood sample or an apheresis of a subject before any number of relevant treatment modalities, including but not limited to treatment with agents such as natalizumab, efalizumab, antiviral agents, chemotherapy, radiation, immunosuppressive agents such as cyclosporine, azathioprine, methotrexate, mycophenolate and FK506, antibodies or other immunoablatives such as CAMPATH, anti-CD3 antibodies, cytoxane, fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, and irradiation. These drugs inhibit calcium-dependent calcineurin phosphatase (*e.g.*, cyclosporin and FK506) or inhibit p70S6 kinase that is important for signaling induced by the growth factor (rapamycin) (Liu *et al.*, Cell 66: 807-815, 1991; Henderson *et al.*, Immun 73: 316-321, 1991, Bierer *et al.*, Curr. Opin. Immun., 5: 763-773, 1993). The cells may be isolated from a patient and frozen for later use together with (*e.g.*, before, simultaneously or after) bone marrow or stem cell transplant, therapy with T lymphocyte ablation using chemotherapeutic agents such as fludarabine, radiotherapy external beam (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. As described herein, the cells may be isolated before and can be frozen for later use in the treatment after therapy with ablation of B lymphocytes, such as agents that react with CD20, for example, Rituxan.

Either before or after the genetic modification of lymphocytes (*e.g.*, T lymphocytes) to express a desirable transgene, lymphocytes can be activated and expanded generally using methods such as those described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and the publication of US patent application. No. 20060121005.

b. Vectors

Transgenes can be introduced into lymphoid cells using various methods. These methods include, but are not limited to, transduction of cells using integration-competent gamma-retroviruses or lentivirus, and DNA transposition.

A wide variety of vectors can be used for the expression of the transgenes. The ability of certain viruses to infect cells or enter cells via receptor-mediated endocytosis, and to integrate into a host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign nucleic acids into cells. Accordingly, in certain embodiments, a viral vector is used to introduce a nucleotide sequence encoding one or more transgenes or fragment thereof into a host cell for expression. The viral vector may comprise a nucleotide sequence encoding one or more transgenes or fragment thereof operably linked to one or more control sequences, for example, a promoter. Alternatively, the viral vector may not contain a control sequence and will instead rely on a control sequence within the host cell to drive expression of the transgenes or fragment thereof. Non-limiting examples of viral vectors that may be used to deliver a nucleic acid include adenoviral vectors, AAV vectors, and retroviral vectors.

For example, an adeno-associated virus (AAV) can be used to introduce a nucleotide sequence encoding one or more transgenes or fragment thereof into a host cell for expression. AAV systems have been described previously and are generally well known in the art (Kelleher and Vos, *Biotechniques*, 17(6):1110-7, 1994; Cotten *et al.*, *Proc Natl Acad Sci USA*, 89(13):6094-6098, 1992; Curiel, *Nat Immun*, 13(2-3):141-64, 1994; Muzyczka, *Curr Top Microbiol Immunol*, 158:97-129, 1992). Details concerning the generation and use of rAAV vectors are described, for example, in U.S. Pat. Nos. 5,139,941 and 4,797,368, each incorporated herein by reference in its entirety for all purposes.

In some embodiments, a retroviral expression vector can be used to introduce a nucleotide sequence encoding one or more transgenes or fragment thereof into a host cell for expression. These systems have been described previously and are generally well known in the art (Nicolas and Rubinstein, In, Rodriguez and Denhardt, eds., Stoneham: Butterworth, pp. 494-513, 1988; Temin, In: *Gene Transfer*, Kucherlapati (ed.), New York: Plenum Press, pp. 149-188, 1986). Examples of vectors for eukaryotic expression in mammalian cells include AD5, pSVL, pCMV, pRc/RSV, pcDNA3, pBPV, etc., and vectors derived from viral systems such as vaccinia virus, adeno-associated viruses, herpes viruses, retroviruses, etc., using promoters such as CMV, SV40, EF-1, UbC, RSV, ADV, BPV, and β -actin.

Combinations of retroviruses and an appropriate packaging line may also find use, where the capsid proteins will be functional for infecting the target cells. Usually, the cells and viruses

will be incubated for at least about 24 hours in the culture medium. The cells are then allowed to grow in the culture medium for short intervals in some applications, *e.g.*, 24-73 hours, or for at least two weeks, and may be allowed to grow for five weeks or more, before analysis. Commonly used retroviral vectors are “defective,” *i.e.*, unable to produce viral proteins required for productive infection. Replication of the vector requires growth in the packaging cell line. The host cell specificity of the retrovirus is determined by the envelope protein, env (p120). The envelope protein is provided by the packaging cell line. Envelope proteins are of at least three types, ecotropic, amphotropic, and xenotropic. Retroviruses packaged with ecotropic envelope protein, *e.g.*, MMLV, are capable of infecting most murine and rat cell types. Ecotropic packaging cell lines include BOSC23. Retroviruses bearing amphotropic envelope protein, *e.g.*, 4070A, are capable of infecting most mammalian cell types, including human, dog, and mouse. Amphotropic packaging cell lines include PA12 and PA317. Retroviruses packaged with xenotropic envelope protein, *e.g.*, AKR env, are capable of infecting most mammalian cell types, except murine cells. The vectors may include genes that must later be removed, *e.g.*, using a recombinase system such as Cre/Lox, or the cells that express them destroyed, *e.g.*, by including genes that allow selective toxicity such as herpesvirus TK, BCL-xs, etc. Suitable inducible promoters are activated in a desired target cell type, either the transfected cell or progeny thereof.

Non-limiting examples of the vectors useful for the present invention include retroviral vector SFG.MCS, and helper plasmids RD114, Peg-Pam3 (Arber *et al.* J Clin Invest 2015 Jan 2; 125(1): 157-168), lentiviral vector pRRL, and helper plasmids R8.74 and pMD2G (*e.g.*, Addgene Plasmid #12259). In some embodiments, the Sleeping Beauty transposon system can be used (Deniger *et al.* 2016 Mol Ther. Jun;24(6):1078-1089). In some embodiments, transgenes can be introduced into cells via deforming a cell as it passes through a small opening, disrupting the cell membrane and allowing material to be inserted into the cell, for example, electroporation (Xiaojun *et al.* 2017 Protein Cell, 8(7): 514-526), or the Cell Squeeze® method. Such electroporation methods of an RNA encoding a transgene allow for transient expression of such transgene in cells which can limit toxicity and other undesirable effects of engineered cells (Barrett *et al.* 2011 Hum Gene Ther. Dec; 22 (12): 1575-1586).

In some embodiments, genome-editing techniques, such as CRISPR/Cas9 systems, designer zinc fingers, transcription activator-like effectors (TALEs), or homing meganucleases are available to induce expression of the transgenes in an immune cell. In general, “CRISPR/Cas9

system” refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated (“Cas”) genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (*e.g.*, tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a “direct repeat” and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a “spacer” in the context of an endogenous CRISPR system), or other sequences and transcripts from a CRISPR locus. One or more elements of a CRISPR system may be derived from a type I, type II, or type III CRISPR system. Alternatively, one or more elements of a CRISPR system may be derived from a particular organism comprising an endogenous CRISPR system, such as *Streptococcus pyogenes*. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system).

In some embodiments, the genetic modification is introduced by transfecting the lymphocyte cell with a vector (*e.g.*, lentiviral vector) encoding one or more transgenes or a functional fragment thereof and CA9 or a functional fragment thereof. In some embodiments, one or more transgenes or a functional fragment thereof and CA9 or a functional fragment thereof can be introduced into the immune cell using one, two, or more vectors.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising exogenous vectors and/or nucleic acids are well known in the art. See, for example, Sambrook *et al.* (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as an *in vitro* and *in vivo* release vehicle is a liposome (*e.g.*, an artificial membrane vesicle).

In the case where a non-viral delivery system is used, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (*in vitro*, *ex vivo*, or *in vivo*). In another aspect, the nucleic acid may be associated

with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, bound to a liposome via a binding molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, in a complex with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, content or in a complex with a micelle, or associated otherwise with a lipid. The compositions associated with lipids, lipids/DNA or lipids/expression vector are not limited to any particular structure in solution. For example, they can be present in a bilayer structure, as micelles, or with a “collapsed” structure. They can also be simply interspersed in a solution, possibly forming aggregates that are not uniform in size or shape.

Lipids are fatty substances that can be natural or synthetic lipids. For example, lipids include fatty droplets that occur naturally in the cytoplasm as well as the class of compounds containing long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine (“DMPC”) can be obtained from Sigma, St. Louis, MO; Dicaprylphosphate (“DCP”) can be obtained from K & K Laboratories (Plainview, NY); Cholesterol (“Choi”) can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol (“DMPG”) and other lipids can be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Lipid stock solutions in chloroform or chloroform/methanol can be stored at about -20°C. Chloroform is used as the sole solvent since it evaporates more easily than methanol. “Liposome” is a generic term that encompasses a variety of unique and multilamellar lipid vehicles formed by the generation of bilayers or closed lipid aggregates. Liposomes can be characterized as having vesicular structures with a bilayer membrane of phospholipids and an internal aqueous medium. Multilamellar liposomes have multiple layers of lipids separated by an aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and trap dissolved water and solutes between the lipid bilayers (Ghosh *et al.*, 1991 *Glycobiology* 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also included. For example, lipids can assume a micellar structure or simply exist as nonuniform aggregates of lipid molecules. Lipofectamine-nucleic acid complexes are also contemplated.

Regardless of the method used to introduce exogenous nucleic acids into a host cell, the presence of the recombinant DNA sequence in the host cell can be confirmed by a series of tests. Such assays include, for example, “molecular biology” assays well known to those skilled in the art, such as Southern and Northern blot, RT-PCR and PCR; biochemical assays, such as the
5 detection of the presence or absence of a particular peptide, for example, by immunological means (ELISA and Western blot) or by assays described herein to identify agents that are within the scope of the invention.

C. METHODS OF TREATMENT

This disclosure further provides a method of treating cancer/tumor or chronic infection.
10 The method comprises administering a therapeutically effective amount of a composition or a pharmaceutical composition, as described above, to a subject in need thereof.

As used herein, the terms “subject” and “patient” are used interchangeably irrespective of whether the subject has or is currently undergoing any form of treatment. As used herein, the terms “subject” and “subjects” may refer to any vertebrate, including, but not limited to, a mammal
15 (*e.g.*, cow, pig, camel, llama, horse, goat, rabbit, sheep, hamsters, guinea pig, cat, dog, rat, and mouse, a non-human primate (for example, a monkey, such as a cynomolgus monkey, chimpanzee, etc.) and a human). The subject may be a human or a non-human. In more exemplary aspects, the mammal is a human.

In some embodiments, the subject is a human. In some embodiments, the subject has a
20 cancer. In some embodiments, the subject is immune-depleted.

As used to describe the present invention, “cancer,” “tumor,” and “malignancy” all relate equivalently to hyperplasia of a tissue or organ. If the tissue is a part of the lymphatic or immune system, malignant cells may include non-solid tumors of circulating cells. Malignancies of other tissues or organs may produce solid tumors. The methods of the present invention may be used in
25 the treatment of lymphatic cells, circulating immune cells, and solid tumors.

Cancers that can be treated include tumors that are not vascularized or are not substantially vascularized, as well as vascularized tumors. Cancers may comprise non-solid tumors (such as hematologic tumors, *e.g.*, leukemias and lymphomas) or may comprise solid tumors. The types of cancers to be treated with the compositions of the present invention include, but are not limited to,

carcinoma, blastoma and sarcoma, and certain leukemias or malignant lymphoid tumors, benign and malignant tumors and malignancies, *e.g.*, sarcomas, carcinomas, and melanomas. Also included are adult tumors/cancers and pediatric tumors/cancers.

Hematologic cancers are cancers of the blood or bone marrow. Examples of hematologic (or hematogenous) cancers include leukemias, including acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia, promyelocytic, myelomonocytic, monocytic, and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high-grade forms), myeloma Multiple, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia, and myelodysplasia.

Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors can be benign or malignant. The different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and lymphomas). Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma and other sarcomas, synovium, mesothelioma, Ewing tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancer, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, carcinoma of the sweat gland, medullary thyroid carcinoma, papillary thyroid carcinoma, sebaceous gland carcinoma of pheochromocytomas, carcinoma papillary, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as glioma) (such as brainstem glioma and mixed gliomas), glioblastoma (also astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, and brain metastasis).

In some embodiments, the cancer is selected from melanoma, sarcoma, ovarian cancer, prostate cancer, lung cancer, bladder cancer, MSI-high tumors, head and neck tumors, kidney cancer, and breast cancer.

The pharmaceutical compositions, as described, can be administered in a manner appropriate to the disease to be treated (or prevented). The amount and frequency of administration will be determined by factors such as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages can be determined by clinical trials.

When “an immunologically effective amount,” “an effective antitumor quantity,” “an effective tumor-inhibiting amount” or “therapeutic amount” is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician having account for individual differences in age, weight, tumor size, extent of infection or metastasis, and patient's condition (subject). It can generally be stated that a pharmaceutical composition comprising the lymphocytes described herein can be administered at a dose of 10^4 to 10^9 cells/kg body weight, *e.g.*, 10^5 to 10^6 cells/kg body weight, including all values integers within these intervals. The lymphocyte compositions can also be administered several times at these dosages. The cells can be administered using infusion techniques that are commonly known in immunotherapy (*see*, for example, Rosenberg *et al.*, New Eng. J. of Med. 319: 1676, 1988). The optimal dose and treatment regimen for a particular patient can be readily determined by one skilled in the art of medicine by monitoring the patient for signs of the disease and adjusting the treatment accordingly.

The administration of the present compositions can be carried out in any convenient way, including infusion or injection (*i.e.*, intravenous, intrathecal, intramuscular, intraluminal, intratracheal, intraperitoneal, or subcutaneous), transdermal administration, or other methods known in the art. Administration can be once every two weeks, once a week, or more often, but the frequency may be decreased during a maintenance phase of the disease or disorder. In some embodiments, the composition is administered by intravenous infusion.

In certain cases, the cells activated and expanded using the methods described herein, or other methods known in the art wherein the lymphocytes are expanded to therapeutic levels, are administered to a patient together with (*e.g.*, before, simultaneously or after) any number of relevant treatment modalities. Also described herein, the lymphocytes can be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablating agents such as CAMPATH, anti-cancer antibodies. CD3 or other antibody therapies, cytoxine, fludarabine,

cyclosporine, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation.

The compositions of the present invention can also be administered to a patient together with (*e.g.*, before, simultaneously or after) bone marrow transplantation, therapy with T lymphocyte ablation using chemotherapy agents such as fludarabine, radiation therapy external beam (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. Also described herein, the compositions can be administered after ablative therapy of B lymphocytes, such as agents that react with CD20, for example, Rituxan. For example, subjects may undergo standard treatment with high-dose chemotherapy, followed by transplantation of peripheral blood stem cells. In certain cases, after transplantation, the subjects receive an infusion of the expanded lymphocytes, or the expanded lymphocytes are administered before or after surgery.

In some embodiments, the method may further include administering to the subject a second therapeutic agent or therapy. The second therapeutic agent is an anti-cancer or anti-tumor agent. In some embodiments, the composition is administered to the subject before, after, or concurrently with the second therapeutic agent or therapy, including chemotherapeutic agents and immunotherapeutic agents.

In some embodiments, the method further comprises administering a therapeutically effective amount of an immune checkpoint modulator. Examples of the immune checkpoint modulator may include PD1, PDL1, CTLA4, TIM3, LAG3, and TRAIL. The checkpoint modulators may be administered simultaneously, separately, or concurrently with the composition of the present invention.

A “chemotherapeutic agent” is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXANTM); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, methyldopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin

(including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratiastatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as the enediyne antibiotics (*e.g.* calicheamicin, see, *e.g.*, Agnew Chem. Intl. Ed. Engl. 33:183-186 (1994); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromomorphores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabacin, caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.* paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (TAXOTERE®, Rhone-Poulenc Rorer, Antony,

France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens, including, for example, tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, xeloda, gemcitabine, KRAS mutation covalent inhibitors and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Additional examples include irinotecan, oxaliplatinum, and other standard colon cancer regimens.

An “immunotherapeutic agent” may include a biological agent useful in the treatment of cancer. In some embodiments, the immunotherapeutic agent may include an immune checkpoint inhibitor (*e.g.*, an inhibitor of PD-1, PD-L1, TIM-3, LAG-3, VISTA, DKG- α , B7-H3, B7-H4, TIGIT, CTLA-4, BTLA, CD160, TIM1, IDO, LAIR1, IL-12, or combinations thereof). Examples of immunotherapeutic agents include atezolizumab, avelumab, blinatumomab, daratumumab, cemiplimab, durvalumab, elotuzumab, lahervarepvec, ipilimumab, nivolumab, obinutuzumab, ofatumumab, pembrolizumab, cetuximab, and talimogene.

D. DEFINITIONS

To aid in understanding the detailed description of the compositions and methods according to the disclosure, a few express definitions are provided to facilitate an unambiguous disclosure of the various aspects of the disclosure. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: 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.

As used herein, “expression” refers to the process by which a polynucleotide is transcribed from a DNA template (such as into an mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as “gene product.” If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

As used herein, the term “recombinant” refers to a cell, microorganism, nucleic acid molecule or vector that has been modified by the introduction of an exogenous nucleic acid molecule or has controlled expression of an endogenous nucleic acid molecule or gene. , Deregulated or altered to be constitutively altered, such alterations or modifications can be introduced by genetic engineering. Genetic alteration includes, for example, modification by introducing a nucleic acid molecule encoding one or more proteins or enzymes (which may include an expression control element such as a promoter), or addition, deletion, substitution of another nucleic acid molecule. , Or other functional disruption of, or functional addition to, the genetic material of the cell. Exemplary modifications include modifications in the coding region of a heterologous or homologous polypeptide derived from the reference or parent molecule or a functional fragment thereof.

By “transgene” or “therapeutic transgene,” it is meant a molecule selected from a soluble receptor, a decoy, a decoy receptor, a dominant negative, a microenvironment modulator, an enzyme, an oxidoreductase, a transferase, a hydrolases, a lysases, an isomerase, a translocase, a kinase, a transporter, a modifier, a molecular chaperone, an ion channel, an antibody, a cytokine, a growth factor, a chemokine, a hormone, a DNA, a ribozyme, a biosensor, an epigenetic modifier, a transcriptional factor, a coding RNA, a non-coding RNA, a small-RNA, a long-RNA, an IRES element, or an exosomal-shuttle RNA.

The term “functional variant” as used herein refers to a modified transgene having substantial or significant sequence identity or similarity to a wild type transgene, such functional

variant retaining the biological activity of the wild type transgene of which it is a variant. In some embodiments, functional variants of transgenes are used.

The term “antigen recognizing receptor,” as used herein, refers to a receptor that is capable of activating an immune cell (*e.g.*, a T-cell) in response to antigen binding. Exemplary antigen recognizing receptors may be native or genetically engineered TCRs, or genetically engineered TCR-like mAbs (Hoydahl *et al.* Antibodies 2019 8:32) or CARs in which a tumor antigen-binding domain is fused to an intracellular signaling domain capable of activating an immune cell (*e.g.*, a T-cell). T-cell clones expressing native TCRs against specific cancer antigens have been previously disclosed (Traversari *et al.*, J Exp Med, 1992 176:1453-7; Ottaviani *et al.*, Cancer Immunol Immunother, 2005 54:1214-20; Chaux *et al.*, J Immunol, 1999 163:2928-36; Luiten and van der Bruggen, Tissue Antigens, 2000 55:149-52; van der Bruggen *et al.*, Eur J Immunol, 1994 24:3038-43; Huang *et al.*, J Immunol, 1999 162:6849-54; Ma *et al.*, Int J Cancer, 2004 109:698-702; Ebert *et al.*, Cancer Res, 2009 69:1046-54; Ayyoub *et al.* J Immunol 2002 168:1717-22; Chaux *et al.*, European Journal of Immunology, 2001 31:1910-16; Wang *et al.*, Cancer Immunol Immunother, 2007 56:807-18; Schultz *et al.*, Cancer Research, 2000 60:6272-75; Cesson *et al.*, Cancer Immunol Immunother, 2010 60:23-25; Zhang *et al.*, Journal of Immunology, 2003 171:219-25; Gnjjatic *et al.*, PNAS, 2003 100:8862-67; Chen *et al.*, PNAS, 2004). In one embodiment, such TCRs can be sequenced and genetically engineered into TILs for use in adoptive cell therapy. In certain aspects, TCRs that recognize MAGE-A1 antigen, MAGE –A3 antigen, MAGE A-10 antigen, MAGE-C2 antigen, NY-ESO-1 antigen, SSX2 antigen, and MAGE-A12 antigen can be genetically engineered into TILs for use in adoptive cell therapy. In yet other embodiments, genetically engineered TILs with TCRs are further engineered to secrete transgenes. In yet other embodiments, CARs are used. In other embodiments, CARs are further engineered to secrete transgenes.

As used herein, the term “antibody” means not only intact antibody molecules, but also fragments of antibody molecules that retain immunogen-binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. Accordingly, as used herein, the term “antibody” means not only intact immunoglobulin molecules but also the well-known active fragments f(ab')₂, and fab. F(ab')₂, and fab fragments that lack the Fe fragment of intact antibody, clear more rapidly from the circulation and may have less non-specific tissue binding of an intact antibody (Wahl *et al.*, J. Nucl. Med. 24:316-325 (1983). The antibodies of the

invention comprise whole native antibodies, bispecific antibodies; chimeric antibodies; fab, fab', single-chain v region fragments (scFv), fusion polypeptides, and unconventional antibodies.

As used herein, the term “single-chain variable fragment” or “scFv” is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of an immunoglobulin covalently linked to form a VH:VL heterodimer. The heavy (VH) and light chains (VL) are either joined directly or joined by a peptide-encoding linker (*e.g.*, 10, 15, 20, 25 amino acids), which connects the n-terminus of the VH with the C-terminus of the VL, or the C-terminus of the VH with the N-terminus of the VL. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility. Despite removal of the constant regions and the introduction of a linker, scFv proteins retain the specificity of the original immunoglobulin. Single-chain Fv polypeptide antibodies can be expressed from a nucleic acid including VH- and VL-encoding sequences as described by Huston, *et al.* (Proc. Nat. Acad. Sci., 85:5879-5883, 1988). See, also, US. Pat. Nos. 5,091,513, 5,132,405 and 4,956,778; and US patent publication nos. 20050196754 and 20050196754. Antagonistic scFvs having inhibitory activity have been described (see, *e.g.*, Zhao *et al.*, Hybridoma (Larchmont) 2008 27(6):455-51; Peter *et al.*, J cachexia sarcopenia muscle 2012 Aug. 12; Shieh *et al.*, J Immunol 2009 183(4):2277-85; Giomarelli *et al.*, Thromb Haemost 2007 97(6):955-63; Fife *et al.*, J Clin Invest 2006 116(8):2252-61; Brocks *et al.*, Immunotechnology 1997 3(3):173-84; Moosmayer *et al.*, Ther Immunol 1995 2(10):31-40). Agonistic scFvs having stimulatory activity have been described (see, *e.g.*, Peter *et al.*, J Bioi Chern 2003 25278(38):36740-7; Xie *et al.*, Nat Biotech 1997 15(8):768-71; Ledbetter *et al.*, Crit Rev Immunol 1997 17(5-6):427-55; Ho *et al.*, Biochim Biophys Acta 2003 1638(3):257-66).

“Treating” or “treatment” as used herein refers to administration of a compound or agent to a subject who has a disorder with the purpose to cure, alleviate, relieve, remedy, delay the onset of, prevent, or ameliorate the disorder, the symptom of a disorder, the disease state secondary to the disorder, or the predisposition toward the disorder.

The term “eliciting” or “enhancing” in the context of an immune response refers to triggering or increasing an immune response, such as an increase in the ability of immune cells to target and/or kill cancer cells or to target and/or kill pathogens and pathogen-infected cells (*e.g.*, EBV-positive cancer cells).

The term “immune response,” as used herein, refers to any type of immune response, including, but not limited to, innate immune responses (*e.g.*, activation of Toll receptor signaling cascade), cell-mediated immune responses (*e.g.*, responses mediated by T cells (*e.g.*, antigen-specific T cells) and non-specific cells of the immune system) and humoral immune responses (*e.g.*, responses mediated by B cells (*e.g.*, via generation and secretion of antibodies into the plasma, lymph, and/or tissue fluids). The term “immune response” is meant to encompass all aspects of the capability of a subject's immune system to respond to antigens and/or immunogens (*e.g.*, both the initial response to an immunogen (*e.g.*, a pathogen) as well as acquired (*e.g.*, memory) responses that are a result of an adaptive immune response).

As used herein, the term “*in vitro*” refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, etc., rather than within a multi-cellular organism.

As used herein, the term “*in vivo*” refers to events that occur within a multi-cellular organism, such as a non-human animal.

The term “disease” as used herein is intended to be generally synonymous and is used interchangeably with, the terms “disorder” and “condition” (as in medical condition), in that all reflect an abnormal condition of the human or animal body or of one of its parts that impairs normal functioning, is typically manifested by distinguishing signs and symptoms, and causes the human or animal to have a reduced duration or quality of life.

The terms “decrease,” “reduced,” “reduction,” “decrease,” or “inhibit” are all used herein generally to mean a decrease by a statistically significant amount. However, for avoidance of doubt, “reduced,” “reduction” or “decrease” or “inhibit” means a decrease by at least 10% as compared to a reference level, for example, a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% decrease (*e.g.* absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level.

As used herein, the term “modulate” is meant to refer to any change in biological state, *i.e.*, increasing, decreasing, and the like.

The terms “increased,” “increase” or “enhance” or “activate” are all used herein to generally mean an increase by a statically significant amount; for the avoidance of any doubt, the terms “increased,” “increase” or “enhance” or “activate” means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%,
5 or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level.

10 The term “effective amount,” “effective dose,” or “effective dosage” is defined as an amount sufficient to achieve or at least partially achieve a desired effect. A “therapeutically effective amount” or “therapeutically effective dosage” of a drug or therapeutic agent is any amount of the drug that, when used alone or in combination with another therapeutic agent, promotes disease regression evidenced by a decrease in severity of disease symptoms, an increase
15 in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. A “prophylactically effective amount” or a “prophylactically effective dosage” of a drug is an amount of the drug that, when administered alone or in combination with another therapeutic agent to a subject at risk of developing a disease or of suffering a recurrence of disease, inhibits the development or recurrence of the disease. The
20 ability of a therapeutic or prophylactic agent to promote disease regression or inhibit the development or recurrence of the disease can be evaluated using a variety of methods known to the skilled practitioner, such as in human subjects during clinical trials, in animal model systems predictive of efficacy in humans, or by assaying the activity of the agent in *in vitro* assays.

Doses are often expressed in relation to bodyweight. Thus, a dose which is expressed as
25 [g, mg, or other unit]/kg (or g, mg etc.) usually refers to [g, mg, or other unit] “per kg (or g, mg etc.) bodyweight,” even if the term “bodyweight” is not explicitly mentioned.

The term “agent” is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule (such as a nucleic acid, an antibody, a protein or portion thereof, *e.g.*, a peptide), or an extract made from biological materials such as bacteria, plants, fungi,
30 or animal (particularly mammalian) cells or tissues. The activity of such agents may render it

suitable as a “therapeutic agent,” which is a biologically, physiologically, or pharmacologically active substance (or substances) that acts locally or systemically in a subject.

The terms “therapeutic agent,” “therapeutic capable agent,” or “treatment agent” are used interchangeably and refer to a molecule or compound that confers some beneficial effect upon administration to a subject. The beneficial effect includes enablement of diagnostic determinations; amelioration of a disease, symptom, disorder, or pathological condition; reducing or preventing the onset of a disease, symptom, disorder or condition; and generally counteracting a disease, symptom, disorder or pathological condition.

“Combination” therapy, as used herein, unless otherwise clear from the context, is meant to encompass administration of two or more therapeutic agents in a coordinated fashion, and includes, but is not limited to, concurrent dosing. Specifically, combination therapy encompasses both co-administration (*e.g.*, administration of a co-formulation or simultaneous administration of separate therapeutic compositions) and serial or sequential administration, provided that administration of one therapeutic agent is conditioned in some way on administration of another therapeutic agent. For example, one therapeutic agent may be administered only after a different therapeutic agent has been administered and allowed to act for a prescribed period of time. See, *e.g.*, Kohrt *et al.* (2011) *Blood* 117:2423.

“Sample,” “test sample,” and “patient sample” may be used interchangeably herein. The sample can be a sample of, serum, urine plasma, amniotic fluid, cerebrospinal fluid, cells (*e.g.*, antibody-producing cells) or tissue. Such a sample can be used directly as obtained from a patient or can be pre-treated, such as by filtration, distillation, extraction, concentration, centrifugation, inactivation of interfering components, addition of reagents, and the like, to modify the character of the sample in some manner as discussed herein or otherwise as is known in the art. The terms “sample” and “biological sample” as used herein generally refer to a biological material being tested for and/or suspected of containing an analyte of interest such as antibodies. The sample may be any tissue sample from the subject. The sample may comprise protein from the subject.

The terms “inhibit” and “antagonize,” as used herein, mean to reduce a molecule, a reaction, an interaction, a gene, an mRNA, and/or a protein’s expression, stability, function or activity by a measurable amount or to prevent entirely. Inhibitors are compounds that, *e.g.*, bind to, partially or totally block stimulation, decrease, prevent, delay activation, inactivate, desensitize,

or down-regulate a protein, a gene, and mRNA stability, expression, function and activity, *e.g.*, antagonists.

“Parenteral” administration of a composition includes, *e.g.*, subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

5 As used herein, the term “pharmaceutical composition” refers to a mixture of at least one compound useful within the invention with other chemical components, such as carriers, stabilizers, diluents, dispersing agents, suspending agents, thickening agents, and/or excipients. The pharmaceutical composition facilitates administration of the compound to an organism.

10 As used herein, the term “pharmaceutically acceptable” refers to a material, such as a carrier or diluent, which does not abrogate the biological activity or properties of the composition, and is relatively non-toxic, *i.e.*, the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

The term “pharmaceutically acceptable carrier” includes a pharmaceutically acceptable
15 salt, pharmaceutically acceptable material, composition or carrier, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a compound(s) of the present invention within or to the subject such that it may perform its intended function. Typically, such compounds are carried or transported from one organ, or portion of the body, to another organ, or portion of the body. Each salt or carrier must be “acceptable” in the
20 sense of being compatible with the other ingredients of the formulation, and not injurious to the subject. Some examples of materials that may serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and
25 suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer’s solution; ethyl alcohol; phosphate buffer solutions; diluent; granulating agent;
30 lubricant; binder; disintegrating agent; wetting agent; emulsifier; coloring agent; release agent;

coating agent; sweetening agent; flavoring agent; perfuming agent; preservative; antioxidant; plasticizer; gelling agent; thickener; hardener; setting agent; suspending agent; surfactant; humectant; carrier; stabilizer; and other non-toxic compatible substances employed in pharmaceutical formulations, or any combination thereof. As used herein, “pharmaceutically acceptable carrier” also includes any and all coatings, antibacterial and antifungal agents, and absorption delaying agents, and the like that are compatible with the activity of the compound, and are physiologically acceptable to the subject. Supplementary active compounds may also be incorporated into the compositions.

It is noted here that, as used in this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise.

The terms “including,” “comprising,” “containing,” or “having” and variations thereof are meant to encompass the items listed thereafter and equivalents thereof as well as additional subject matter unless otherwise noted.

The phrases “in one embodiment,” “in various embodiments,” “in some embodiments,” and the like are used repeatedly. Such phrases do not necessarily refer to the same embodiment, but they may unless the context dictates otherwise.

The terms “and/or” or “/” means any one of the items, any combination of the items, or all of the items with which this term is associated.

The word “substantially” does not exclude “completely,” *e.g.*, a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

As used herein, the term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In some embodiments, the term “approximately” or “about” refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value). Unless indicated otherwise herein, the term “about” is intended to include values, *e.g.*,

weight percents, proximate to the recited range that are equivalent in terms of the functionality of the individual ingredient, the composition, or the embodiment.

It is to be understood that wherever values and ranges are provided herein, all values and ranges encompassed by these values and ranges, are meant to be encompassed within the scope of the present invention. Moreover, all values that fall within these ranges, as well as the upper or lower limits of a range of values, are also contemplated by the present application.

As used herein, the term “each,” when used in reference to a collection of items, is intended to identify an individual item in the collection but does not necessarily refer to every item in the collection. Exceptions can occur if explicit disclosure or context clearly dictates otherwise.

The use of any and all examples, or exemplary language (*e.g.*, “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

All methods described herein are performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. In regard to any of the methods provided, the steps of the method may occur simultaneously or sequentially. When the steps of the method occur sequentially, the steps may occur in any order, unless noted otherwise.

In cases in which a method comprises a combination of steps, each and every combination or sub-combination of the steps is encompassed within the scope of the disclosure, unless otherwise noted herein.

Each publication, patent application, patent, and other reference cited herein is incorporated by reference in its entirety to the extent that it is not inconsistent with the present disclosure. Publications disclosed herein are provided solely for their disclosure prior to the filing date of the present invention. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates, which may need to be independently confirmed.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

5 E. EXAMPLES

EXAMPLE 1

This example describes the materials and methods used in the subsequent EXAMPLES.

Mice and cell lines

Female C57BL/6 mice aged 6 weeks were purchased from Harlan (Harlan, Netherlands) and housed at the animal facility at the University of Lausanne (UNIL, Epalinges, Switzerland) in compliance with guidelines. C57BL/6 OT-1 CD45.1+ and C57BL/6 CD8a-/- mice are described in Hogquist KA *et al.* (Hogquist KA *et al.* Cell 76(1):17-27 PubMed: 8287475MGI: J:92867) and Fung-Leung WP *et al.* (Fung-Leung WP *et al.* Cell 65(3):443-9 PubMed: 1673361MGI: J:68956). All *in vivo* experiments were conducted in accordance and with approval from the Service of Consumer and Veterinary Affairs (SCAV) of the Canton of Vaud, Switzerland.

The B16 melanoma cell line expressing ovalbumin (B16-OVA) was previously generated by retroviral transduction of the B16.F10 cell line purchased from ATCC and was grown as a monolayer in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 µg/ml streptomycin sulfate. Cells were passaged twice weekly to maintain them under exponential growth conditions and were routinely tested for mycoplasma contamination. The Phoenix Eco retroviral ecotropic packaging cell line, derived from immortalized normal human embryonic kidney (HEK) cells, was maintained in RPMI 1640-Glutamax media supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate.

Human embryonic kidney (HEK) 293T cells were purchased from the ATCC (CRL-3216) and cultured in RPMI 1640 Glutamax medium (Invitrogen), 10% FBS (heat-inactivated for 30 min at 56°C; Gibco), 1% Penicillin/Streptomycin (ThermoFisher Scientific). HEK 293T cells were used to produce retroviral and lentiviral particles. The HLA-A2.1^{pos}/NY-ESO^{pos} melanoma cell lines Me275 and A375, and the HLA-A2.1^{pos}/NY-ESO^{neg} cell line NA8 (obtained from the UNIL

Department of Oncology) were cultured in IMDM supplemented with 10% FBS and 1% Penicillin/Streptomycin.

Design of Bi-Cistronic Expression Cassettes

The retroviral vector pMSGV1 (murine stem cell virus (MSCV)-based splice-gag vector) comprising the MSCV long terminal repeat (LTR) was used as the backbone for all the constructs. Expression cassettes typically encoded the signal peptide of a murine IgG Kappa Chain region V-III MOPC 321 (*e.g.*, Uniprot ID: P01650) followed by the N-terminal ectodomain of murine PD-1 (*e.g.*, Uniprot ID: Q02242 residues S21-Q167, C83S) fused to human IgG4_Fc (*e.g.*, Uniprot ID: P01861.1, residues P104-K327) referred here as PD-1.IgG4 decoy. The restriction sites AgeI and EcoRI flanked this first part at the 5' and 3' ends, respectively. The second part followed the T2A sequence and was composed by the signal peptide of murine IFN-beta (*e.g.*, Uniprot ID: P01575.1) followed by a gene-string encoding one of the following molecules: murine IL-33 (*e.g.*, Uniprot ID: Q8BVZ5.1, residues S109-I266), murine LIGHT (*e.g.*, Uniprot ID: Q9QYH9.1, residues D72-V239), murine CD40L (*e.g.*, Uniprot ID: P27548, residues M112-L260) and no alpha mutant IL-2 (*e.g.*, Uniprot ID: P60568.1, residues A21-T153, mutations: R58A, F62A, Y65A, E82A, and C145S). The restriction sites MluI and SalI flanked this second part at the 5' and 3' ends, respectively. Consequently, after respective cloning, the following constructs were obtained: PD-1.IgG4_T2A_IL-2^V, PD-1.IgG4_T2A_IL-33, PD-1.IgG4_T2A_LIGHT, and PD-1.IgG4_T2A_CD40L. All genes-strings were murine codon-optimized and synthesized by GeneArt AG, and all constructs were fully sequenced by Microsynth AG after cloning in the MSGV vector.

As shown in FIGS. 3A-C, codon-optimized gene strings encoding the PD1 decoy and truncated EGFR, separated by the picorna virus-derived 2A sequence, as well as the CD40 ligand decoy, and IL-2 variant, were ordered from GeneArt (ThermoFisher Scientific) and cloned into the retroviral vector pSFG (for constitutive expression) or pSFG-SIN (self-inactivating) for activation based gene-expression under NFAT promoter. The vectors were amplified in Stellar competent cells (*E. coli* HST08, #636763, Takara) and purified with plasmid mini/maxi-prep kit (Genomed) upon sequence confirmation (Microsynth AG).

A gene string encoding the HLA/A2:NY-ESO-1 peptide T cell receptor (TCR) comprising TCR α 23 and TCR β 13.1 was ordered from GeneArt (ThermoFisher Scientific). The TCR α and

TCR β chains were codon-optimized and separated by the picorna virus-derived 2A sequence. The gene string was incorporated into the lentiviral vector pRRL, in which most of the U3 region of the 3' long terminal repeat was deleted, resulting in a self-inactivating 3' long terminal repeat (SIN).

Lentivirus Production

10x10⁶ HEK 293T cells were seeded in T150 flasks with RPMI complete medium (RPMI 1640 Glutamax medium (Invitrogen) 10% FBS (Gibco), 1% Penicillin/Streptomycin). Approximately 24 hours later (at 70-80% confluency), and the cells were transfected with 7 μ g pVSV-G (VSV glycoprotein expression plasmid), 18 μ g of μ R874 (Rev and Gag/Pol expression plasmid), and 15 μ g of pRRL transgene plasmid using a mix of 107 μ l of Turbofect and 2 ml of Optimem media (51985026, Invitrogen). After 30 minutes of incubation at room temperature, the DNA mixture was added on top of the cells, and the volume was adjusted up to a total of 30 ml. After 24 hours, the medium was refreshed, and the viral supernatant was harvested at 48 hours post-transfection. The viral particles were concentrated by ultracentrifugation and resuspended in 400 μ l of RPMI complete media. Aliquots of virus of 100-200 μ l per Eppendorf tube were prepared and stored at -80C.

Retrovirus Production

Phoenix Eco cells were seeded at 1×10^7 per T-150 tissue culture flask in 25 ml culture medium 24 hours prior to transfection with 14.4 μ g of pCL-Eco Retrovirus Packaging Vector and 21.4 μ g of pMSGV transfer plasmid using Turbofect (Thermo Fisher Scientific). All plasmids were purified using JETSTAR 2.0 Plasmid Maxiprep Kit (Genomed). For the transfection mixture, a 3:1 ratio of turbofect:plasmid was prepared in 2 ml of Optimem and incubated for 30 minutes at RT. Medium was then removed from T-150 flasks bearing 80-90% confluent Phoenix Eco cells, and the transfection mixture was applied and incubated for 1 minute, followed by addition of 25 ml fresh medium. The viral supernatant was harvested 48 hours post-transfection, followed by addition of 25 ml of fresh media. A second harvest was performed again 24 hours later. The viral particles in both SN were concentrated by ultracentrifugation for 2-hours at 24,000g at 4°C with a Beckman JS-24 rotor (Beckman Coulter) and suspended in 0.5 ml murine T-cell medium, then viral titer was determined. Finally, the retrovirus was aliquoted, frozen on dry ice, and stored at -80°C.

In another example, 10×10^6 HEK 293T cells were seeded in 17 ml RPMI, 10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (ThermoFisher Scientific) in a T150 flask overnight at 37 degrees. The following day (at 85-95% confluency of 293T cells), a mix of 120 μ l turbofect (LifeTechnologies) and 3 ml OptiMem per transfection (per T150 flask) was prepared and then combined with the retroviral plasmids: 22 μ g PamPeg, 7 μ g RDF-RD114, 18 μ g SFG or SFG-SIN encoding the gene of interest. The medium was gently removed from the 293T cells, and the retroviral plasmid mix was pipetted onto the 293T cells. After resting 5 minutes, an additional 16 ml medium was gently added. Cells were incubated at 37°C overnight. The next day, the medium was refreshed, and the day following (at 48 hours), the virus was harvested from the filtered supernatant by ultracentrifugation (2 hours at 24000x g). Fresh medium was added to the 293T cells for a second harvest of virus at 72 hours. Aliquots of virus on both days of 100-200 μ l per Eppendorf tube were prepared and stored at -80C.

Murine T-cell transduction

Primary murine OT-1 cells were isolated from single-cell suspensions of dissociated spleens from CD45.1+ congenic OT-1 C57BL/6 mice aged 6-10 weeks using the Pan T cell Isolation Kit II for the mouse (Miltenyi Biotec cat# 130-095-130) and cultured in RPMI 1640-Glutamax media supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate, 1mM Pyruvate, 50 μ M BME, and 10mM non-essential amino acids (T-cell medium).

The cultures were maintained at a cell density of $0.5-1 \times 10^6$ cells/ml, replenished with fresh T-cell media every other day until day 15 (media was supplemented with 10 IU/ml of human no alpha mutant IL-2 alone until day 3 and then together with 10 ng/ml of hIL-7/IL-15). On day 7, the cell expression of the molecules was assessed by intracellular flow-cytometric analysis, and their presence in the supernatant was assessed by ELISA. Finally, engineered OT-1 T cells were adjusted according to the transduction efficiency of the PD-1.IgG4 decoy prior to cell transfer. Recombinant human IL-7 and human IL-15 were obtained from Miltenyi Biotec.

Isolated naïve OT-1 T cells were plated at 1×10^6 /ml in 24-well plates in T-cell medium and stimulated with α CD-3/ α CD-28 Ab-coated beads (Invitrogen) and 10 IU/ml human no alpha mutant IL-2. Twenty-four hours post-activation, T cells were transduced for the first time with retrovirus at a multiplicity of infection (MOI) of 10. This transduction was performed in non-

tissue culture grade 24-well plates (Becton Dickinson Labware) pre-coated overnight at 4°C with 20 mg/ml of recombinant retronectin (RetroNectin; Takara), washed, blocked with 2% bovine serum albumin (BSA) in PBS for 30-minutes at RT, and then given a final wash. Following addition of the retrovirus (250 µl), the plates were centrifuged at 2000xg for 1.5-hours at 32 °C. 125 µl of supernatant was aspirated, and 1x10⁶ of activated T cells were transferred to each coated well. The plates were centrifuged for 10 min at 1200 rpm and incubated overnight at 37°C, 5% of CO₂. The second transduction was done at 48-hours post activation following the protocol explained above. On day 7, the cell expression of the molecules was assessed by intracellular flow-cytometric analysis, and their presence in the supernatant by ELISA. Finally, engineered OT-1 T cells were adjusted based on the PD-1.IgG4 decoy expression prior to cell transfer.

The cultures were maintained at a cell density of 0.5-1x10⁶ cells/ml and replenished with fresh T-cell media every other day until day 15 following an *in vitro* expansion protocol optimized to generate CD44+CD62L+TCF1+ central memory CD8 T cells. T cell media was supplemented with 10 IU/ml of human no alpha mutant IL-2 alone until day 3 and then together with 10 ng/ml of hIL-7/IL-15 until the end of the culture. Recombinant human IL-7 and human IL-15 were obtained from Miltenyi Biotec.

Human T-cell purification and activation

Healthy donor apheresis and buffy coats were purchased from the Transfusion Interrégionale CRS SA, Epalinges, Switzerland, with written consent under an approved University Institutional Review Board protocol. Peripheral blood mononuclear cells (PBMCs) were prepared using Lymphoprep (StemCell Technologies) density gradient centrifugation, and CD8⁺ or CD4⁺ T cells were negatively isolated using CD8 or CD4 magnetic Microbeads (Miltenyi), following the manufacturer's protocol. Isolated CD8⁺ and CD4⁺ T cells were stimulated with anti-CD3/CD28 beads (Invitrogen) at a 2:1 Beads: T cell ratio in the presence of human IL-2 (GlaxoSmithKline).

Human T-cell lentiviral and retroviral transduction

Lentiviral transduction of T cells was performed 24 hours post-activation by direct addition of the viral particles in the culture medium (MOI 20) and enhanced by concurrent addition of Lentiboost (Sirion Biotech). Retroviral transduction of T cells was performed 48h post-activation. T cells were transferred to retronectin-coated plates previously spinoculated with retroviral

particles at 2000×g for 1.5 hours. T cells were removed from retronectin-coated plates the next day. The antiCD3/antiCD28 beads (Thermo Fisher Scientific) were removed 5 days post-activation, and the T cells were maintained thereafter in RPMI 1640-Glutamax (Thermo Fisher) supplemented with 10% heat-inactivated FBS (Gibco), 1% Penicillin/Streptomycin, 10 ng/ml human IL-7 (Miltenyi), and 10 ng/ml IL-15 (Miltenyi) at 0.5-1x10⁶ T cells/ml.

Human T-cell co-transduction with lentivirus and retrovirus

For co-transduction, human T cells were purified and bead-activated (Per 48-well: 0.5x10⁶ T cells + 1x10⁶ antiCD3/antiCD28 beads + 50IU/ml IL-2) for 18-22 hours prior to the addition of concentrated lentivirus (100 µl), and optionally also 1 µl Lentiboost (Sirion Biotech) to enhance transduction efficiency. The next day, the transduced T cells were transferred to retronectin-coated plates previously spinoculated with retroviral particles at 2000×g for 1.5 hours. The following day, the T cells were transferred to a tissue culture plate. On day five the beads were removed, and the T cells were transferred to larger wells and provided fresh medium supplemented with 10 ng/ml IL-15 and 10 ng/ml IL-7. The provision of fresh medium plus cytokines was performed every 2-3 days. From day 7-10, co-transduction efficiency can be determined by flow cytometry.

Retrovirus transduction of tumor-infiltrating lymphocytes (TILs)

Defrosted TILs were previously expanded from dissociated patient tumor fragments. 0.5x10⁶ TILs were stimulated in 48-well plates in 500 µl RPMI, 10% FBS plus 25 µl GMP-grade TransAct (1:20, Miltenyi Biotech) and 6000 IU/ml IL-2. A non-tissue culture plate was coated with retronectin (Takara Bio, dilute 1mg/ml 50 times, 250 µl per 48well) overnight at 4°C. The next day, the retronectin was removed and blocked with 500 µl of RPMI, 10% FBS (Gibco), 1% Penicillin/Streptomycin for 30 minutes at 37°C. Subsequently, the medium was removed, and 50-100 µl of concentrated retrovirus was added in 50 µl medium, followed by spinning for 1 hour at 2000g at 25°C. Then the supernatant was removed, the TILs were added and spun for 10 minutes at 1000g at 25°C. Incubate overnight at 37°C and then transfer to 48-well tissue culture plates with fresh medium. On day 5, the TILs were transferred to larger well plates and supplemented with fresh medium (RPMI, 10% FBS (Gibco), 1% Penicillin/Streptomycin, 6000IU/ml IL-2). Transduction efficiency was evaluated on day 7-10. From day 5 onwards, fresh medium was provided every 2-3 days.

Flow cytometric analysis

All FACS data were acquired at an LCRII flow cytometer (BD) and analyzed using FlowJo software. The fixable aqua dead dyes L34965 or L34975 (Invitrogen) were used as per manufacturer's instructions for dead cell exclusion. The following antibodies were used for T cell staining: anti-Vb13.1:PE (IM2292, BD Bioscience), anti-IFN γ :PeCy7 (502527, Biolegend). Tetramer (A2/NY-ESO-1₁₅₇₋₁₆₅; produced in-house) staining was used to evaluate TCR transduction efficiency.

Flow Cytometric analysis for evaluating the expression of immunomodulatory factors by gene-engineered T cells

One-week post-transduction gene engineered OT-1 T cells were incubated with 50 μ l of Live/Dead Fixable aqua dead for 30 minutes in PBS at room temperature, washed and then incubated again with 50 μ l of FCR blocking reagent (clone 2.4G2 BD Pharmingen) for 30 minutes at 4°C. Cells were washed again and incubated at 4°C for another 30 minutes with surface markers directed Abs against CD3 (145-2C11, Invitrogen), CD8 α (53-6.7, BioLegend), and CD45.1 (A20, BioLegend). For intracellular staining, the following antibodies were used: anti-human hIgG4-Fc (Abcam, clone: HP6025) for detecting the PD-1.IgG4 decoy and anti-mouse IL-33 (eBioscience, clone: 396118). After surface staining, gene-engineered OT-1 cells were washed twice and fixed/permeabilized using the FoxP3 transcription factor staining buffer set (Invitrogen) according to the manufacturer's recommendations. For the detection of each molecule, the cells were further washed and incubated for 30 minutes with respective antibodies at room temperature. Cells were washed and resuspended in PBS supplemented with 2% BSA and 0.01% azide (FACS buffer) FACS buffer to be acquired with a BD flow cytometer LSRII cytometer and analyzed using FlowJo software v11 (Tree Star Inc.).

Flow cytometric analysis to evaluate intracellular cytokine or PD1-Fc decoy or CD40L decoy production

In order to assess intracellular cytokine production or PD1 decoy or CD40L decoy production via FACS, 50,000 live T cells per well were activated with the combination of plate-coated anti-CD3 (5 μ g/ml) and soluble anti-CD28 (2 μ g/ml) antibody for 7 hours in round-bottom 96-well plates (or with anti-CD3/anti-CD28 beads). To prevent protein secretion, Golgi stop was added (BD Biosciences) at a dilution of 1:400 to the wells 1.5 hours after the initiation of the assay.

A standard fixation/permeabilization kit (BD Biosciences) was used according to manufacturer's instructions to fix and permeabilize the T cells before assessing their transduction efficiency or their capacity to produce the molecule of interest. An anti-Fc antibody was used for detection of the decoys. Antibodies specific for the cytokine of interest (IL-2, IFN- γ) were used.

ELISA for evaluating the secretion of immunomodulatory factors by gene-engineered T cells

One-week post activation and transduction, 106 genetically engineered OT-1 T cells were seeded in 1 ml of serum-free RPMI media for 72 hours. Then SN was harvested and tested for each molecule. For PD1.IgG4, a modified-ELISA with the following setup was used. Plates were coated with anti-mouse PD1 Ab (R&D, AF1021, 2 μ g/ml), incubated with SN and PD1.IgG4 was detected with anti-hIgG4-HRP Ab (Abcam, ab99817, dilution 1:1000).

For IL-2^V, a modified-ELISA with the following setup was used. Plates were coated with anti-human IL-2 Ab (R&D, AF-202-NA, 3 μ g/ml), incubated with supernatant, and IL-2^V was detected by biotinylated polyclonal anti-Human IL-2 Ab (Invitrogen, 13-7028-81, dilution 1:500) followed by streptavidin-HRP (BioLegend, dilution 1:1000). SN from OT-1 T cell transduced for expressing either the fusion molecule TIM-3.IgG4 or IL-2^V were used as negative controls. For detection of LIGHT, IL-33, and CD40L, three commercial ELISA kits were used: mouse LIGHT/TNFSF14 DuoSet ELISA developed by R&D (DY1794-05), LEGEND MAXTM Mouse IL-33 ELISA Kit developed by BioLegend (436407), Mouse CD40Ligand/TNFSF5 ELISA Kit developed by Novus Biological (NBP1-92662).

Adoptive cell transfer in tumor-bearing mice

B16-OVA tumor cells were harvested with 0.05% trypsin, washed, and resuspended in PBS for injection. 1×10^5 tumor cells were injected subcutaneously in the right flank of C57BL/6 mice, aged 7 weeks. On day 11 (average tumor volume 100-200 mm³), mice were regrouped in order to have comparative average tumor volumes between experimental arms, with $n \geq 5$ mice/group. On day 12 and 15 mice were treated with i.v transfer of 5×10^6 gene-engineered CD44+ CD62L+ TCF1+ OT-1 T cells or control non-transduced OT-1. Mice were monitored three times/week, and tumor length (L; greatest longitudinal measurement) and width (W; greatest transverse measurement) measured by caliper by an independent investigator in a blinded manner. Tumor volumes (V) were calculated using the formula: $V = (L \times W^2)/2$. The average tumor

volumes/group were plotted $\pm$ SD. Mice were sacrificed once tumors reached 1000mm³, or, according to regulation, if they became distressed or moribund.

ELISA for evaluating the secretion of immunomodulatory factors by gene-engineered T cells.

One-week post-transduction 1×10^6 gene-engineered OT-1 T cells were seeded in a 24-well plate in 1 ml of serum-free RPMI media for 72 hours. SN was then harvested and tested for each molecule by ELISA. PD1.IgG4 homemade-ELISA: coating Ab: anti-mouse PD-1 (R&D, AF1021, 2 μ g/ml), Detection Ab: anti-hIgG4-HRP (Abcam, ab99817, dilution 1:1000). IL-2^V home-made ELISA: coating Ab: anti-human IL-2 (R&D, AF-202-NA, 3 μ g/ml), Secondary Ab biotinylated polyclonal anti-Human IL-2 (Invitrogen, 13-7028-81, dilution 1:500), streptavidin-HRP (BioLegend, dilution 1:1000). SN from OT-1 T cell transduced for expressing either the fusion molecule TIM-3.IgG4 or IL-2^V were used as negative controls. For detection of IL-33, a commercial LEGEND MAXTM Mouse IL-33 ELISA Kit developed by BioLegend (436407) was used.

ADCC measured by chromium release assay

Autologous PBMCs were defrosted, placed at a concentration of 1×10^6 /ml, and added in a 6-well plate at 3 ml per well in the presence of 10 ng/ml GM-CSF. The next day, 0.5×10^6 EGFR⁺ T cells were loaded with 50 μ Ci Chromium-51, re-suspended, and put in a 37°C water-bath for approximately 1 hour. The T cells were then washed twice and suspended at a concentration of 400,000 cells/ml, and 50 μ l of the T cells (=2,000 cells) per well was transferred. Cetuximab (anti-EGFR antibody) at 300 μ g/ml or 30 μ g/ml was prepared. 50 μ l of Cetuximab was added to the T cells and incubated for 30 minutes at 37°C. The PBMCs (effector cells) were harvested at 1.2×10^6 cells/ml in RPMI, 10% FBS (Gibco), 1% Penicillin/Streptomycin. 1 in 3 dilutions of the effector PBMCs (1.2×10^6 , 0.4×10^6 , 1.33×10^6 , and 0.42×10^6 PBMC/ml) were prepared, and 50 μ l was added to the wells containing tEGFR⁺ T cells plus anti-EGFR antibody. different ratios of effector :target cells (30:1, 10 :1, 3 :1, 1 :1, in triplicate) were set up. As a positive control, 1x TritonX was added to the T cells (=maximum chromium release). All negative controls (medium only, T cells no PBMCs, T cells plus PBMCs but no antibody, etc.) were set up. The plates were spun at 1500 rpm and placed at 37°C for 4-5 hours. 50 μ l of supernatant was transferred to lumaplate wells and allowed to dry overnight. The following day, chromium levels were evaluated with the topCounter.

Co-Culture assay and ELISA to measure cytokine production

TCR-T cells co-engineered to express the PD1 decoy plus truncated EGFR (and all control T cell conditions) were prepared at a concentration of 1×10^6 TCR⁺ T cells/ml, and tumor cells were prepared at 1×10^6 cells/ml. 100 μ l each of the T cells and the tumor cells were combined in 96-well round-bottom plates. The plates were spun for 1 minute at 1500 rpm and incubated at 37°C for 48-72 hours. Evaluate IFN- γ levels in the supernatant by ELISA (Invitrogen) according to the manufacturer's recommendation.

Co-Culture assay and ELISA for evaluating secretion of PD1 and CD40L decoys

1×10^6 primary UTD and co-transduced T cells (engineered to express the NY TCR and secrete the PD1 decoy plus tEGFR) were co-cultured with 1×10^6 target cells per well in 96-well round bottom plates, in duplicate, in a final volume of 200 μ L complete RPMI media. The plates were spun for 1 minute at 1500 rpm and incubated at 37°C. After 24-hours, the co-culture supernatants were harvested and tested for the presence of PD1-Fc fusion decoy molecules by capture on plate-bound anti-PD1 antibody or plate-bound human PD-L1 protein. The bound PD1-Fc decoy molecules were detected by anti-IgG-Fc Ab. The same conditions were used to evaluate CD40L decoy secreted in the supernatant except that a commercial ELISA kit is used (Invitrogen).

Bacterial production and purification of soluble PD1 decoy proteins

The PD1 decoy genes comprising a 6xHis tag at the C-terminus for purification purposes were cloned into vector pet21b and used to transform chemocompetent Rosetta pRAREII bacterial cells (Sigma-Aldrich). Colonies isolated from LB Ampicillin 150mg/ml-Chloramphenicol 34mg/mL (LBAC) 1.5% Agar plates were used to inoculate 20mL warmed LBAC medium overnight, which was subsequently transferred to 1L warmed LBAC medium. Induction was performed with 0.5mM IPTG 3h00 at 37° prior to centrifugation of the bottle. The bacterial pellets were resuspended in 35 mL final of TH 50mM- NaCl 1M add DDM 0.02%-PMSF 200mM- EDTA 1mM- DTT 1mM (TH 1M=Tris-Hepes 1M Mix Tris Base & Hepes free acid equimolar 1M, pH 8) and lysed on ice by sonication. Following centrifugation (discard supernatant), the resuspension was repeated. The pellet was finally suspended in 50mM TH8.0 NaCl 1M Urea 8M-200mM PMSF-0.02% DDM. After solubilization of the inclusion bodies (30 minutes), 100 mM CuSO₄ was added to the lysate, which was then incubated at room temperature for 30 minutes to allow

disulfide bridge formation. 1/100V of 100% acetic acid was then added to shift pH to 4.5. The sample was then centrifuged, and dialysis was performed on the supernatant. The proteins were then purified on a Ni-NTA column, washed with at least 20 column volumes with TRIS500, and eluted directly with TRIS500-Imidazole500mM. The sample was subsequently concentrated (Amicon, 3 kDa cut-off), centrifuged, and purified on a Superdex75 or 200 Column. The sample was finally concentrated to 10mg/mL with 10% glycerol, aliquots prepared, and flash-frozen for storage at - 80° C.

Immune subsets depletions, checkpoint blockade, and FTY720 treatment.

Specific cellular subsets were depleted by administering 250 µg/dose of depleting antibody i.p. every three days beginning 1 day before therapy: CD4 T cells with α-mouse CD4 (clone GK1.5, BioXCell), NK cells with α-mouse NK1.1 (clone PK136, BioXCell), neutrophils with α-mouse Ly6G (clone 1A8, BioXCell). For checkpoint blockade, mice were injected i.p. every three days with 250 µg/dose of α-mouse PD-L1 (BioXcell, 10F.962) and α-mouse TIM-3 (BioXcell, RMT3-23). To block emigration of lymphocytes from secondary lymph organs, a stock solution of FTY720 (10 mg/ml in DMSO) obtained from SIGMA was prepared and then diluted to 1 mg/ml in water before administration. Finally, 100 µg of the drug was administrated i.p. every three days beginning 2 days before therapy. Both depletions and sequestration (FTY720) of immune cells were confirmed by flow cytometry of PBMC.

Preparation of single tumor-cell suspensions, antibodies for flow cytometry and ex vivo re-stimulation for cytokine production.

Tumors were excised 5 and 12 days after the first adoptive cell transfer and dissociated into a single-cell suspension by combining mechanical dissociation with enzymatic degradation of the extracellular matrix using the commercial Tumor Dissociation kit for mouse (Miltenyi Biotec, 130-096-730). Following the single-cell suspension, 2.5×10^6 live cells were seeded in 96-well plates and incubated with 50 µl of Live/Dead Fixable aqua dead for 30 minutes in PBS at room temperature, then Fc receptors were blocked by incubation for 30 min. at 4°C with 50 µl of purified anti-CD16/CD32 mAb (clone 2.4G2 BD Pharmingen). Cells were then stained for 30 min. at 4°C with the fluorochrome-conjugated mAbs of interest in 50 µl of FACS Buffer. Subsequently, the cells were washed twice and fixed/permeabilized using the FoxP3 transcription factor staining buffer set (Invitrogen) for intracellular staining. Analysis of stained cells was performed using an

LSRII cytometer and FlowJo software.

The following antibodies were used: CD45.1 (clone A20, BioLegend); CD3 (clone 145-2C11, Invitrogen), CD4 (clone GK1.5, BioLegend); CD8 (clone 53.6.7, BioLegend), FOXP3 (clone FJK-16S, Invitrogen), NK1.1 (clone PK136, BioLegend), CD44 (clone IM7, BioLegend),
 5 PD-1 (clone 29F.1A12, BioLegend), LY6C (clone HK1.4, BioLegend), Granzyme C (clone SFC1D8, BioLegend), TCF1 (clone C63D9, Cell Signaling Technology), anti-rabbit IgG (H+L), F(ab')₂ Fragment AF488 or PE conjugated (Cell Signaling Technology), Granzyme B (clone GB11, Novul Biological), CD69 (clone H1.2F3, BioLegend), TIM-3 (clone RMT3-23, BioLegend), CD137/4-1BB (clone 17B5, Invitrogen), KLRG1 (clone 2F1/KLRG1, BioLegend),
 10 KI67 (clone 16A8, BioLegend), IFN γ (clone XM61.L, Invitrogen), TNF α (clone MP6-XT22, BioLegend), TOX (clone TXRX10, Invitrogen), CD45 (clone 30-F11, BioLegend),

Fluorescence minus one (FMO) controls were stained in parallel using the panel of antibodies with sequential omission of one antibody. FMO staining was performed as a control for the following antibodies: TCF1, Ki67, 4-1BB, Granzyme B, TNF α , IFN γ , PD-1, and TIM-3.
 15 Isotype control was used for Granzyme C staining (clone HTK888, BioLegend). Precision Count Beads™ (BioLegend) were used to obtain absolute counts of cells during acquisition on the flow cytometer.

For the detection of cytokine production, single tumor cells suspension (2.5×10^6 live cells) were *in vitro* re-stimulated in 24-well plates with 1 μ g/ml well-coated anti-mouse CD3 (clone
 20 17A2, Invitrogen) and 2 μ g/ml of soluble anti-mouse CD28 (clone 37.51, Invitrogen) for 4h in the presence of Brefeldin A (5 μ g/ml). Cells were surface stained before fixation and permeabilization as described above, which was followed by intracellular staining.

Immunofluorescence labeling and microscopy

For immunohistochemistry analysis, tumor tissues were isolated and were fixed in 1%
 25 PFA in PBS overnight, infiltrated with 30% sucrose the next day (overnight), and then embedded and frozen in OCT compound. Cryostat sections were collected on Superfrost Plus slides (Fisher Scientific), air-dried, and preincubated with a blocking solution containing BSA, normal mouse serum, normal donkey serum (Sigma), and 0.1% triton. Then they were labeled overnight at 4°C with primary antibodies diluted in PBS with 0.1% triton. After washing with PBS with 0.1%
 30 triton, the secondary reagents were diluted in PBS with 0.1% triton and applied for 45 minutes at

RT. Finally, after additional washes with PBS and 0.1% triton, DAPI (Sigma) was used to stain the nuclei, followed by a PBS wash and mounting in DABCO (homemade). Images were acquired with a Zeiss AxioImager Z1 microscope and an AxioCam MRC5 camera. Images were treated using Fiji (NIH) or Adobe Photoshop. Exposure and image processing were identical for mouse groups, which were directly compared.

Antibodies (ab) used for the CD8/CD45.1/CD105 labeling: 1° ab: Rat-a-mouse CD8a (clone 53-6.7), Rabbit-a-mouse CD105 (clone MJ7/18), Mouse-a-mouse CD45.1 Biotin (clone A20.1). 2° reagent: Donkey-a-Rat Alexa 488 (Invitrogen, # A21208), Donkey-a-Rabbit Cy3 (Jackson ImmunoResearch # 711-165-152), Streptavidin APC (Biolegend, #405207).

Antibodies (ab) used for the CD8-CD45.1-TCF1 labeling: 1° ab: Rat-a-mouse CD8a (53-6.7), Rabbit-a-mouse TCF-1 (Cellsignalling. clone C63D9), #2203), Mouse-a-mouse CD45.1 Biotin (clone A20.1). 2° ab: Donkey-a-Rat Alexa 488 (Invitrogen, # A21208), Donkey-a-Rabbit Cy3 (Jackson ImmunoResearch # 711-165-152.), Streptavidin APC (Biolegend, #405207).

Single-cell RNA-seq analysis.

Aggregated UMI counts matrix generated by CellRanger was filtered in order to select high-quality CD8 TIL transcriptomes. First, cells having 500 to 5000 detected genes, 2000 to 30000 UMI counts, mitochondrial content below 5%, and % ribosomal protein content below 50 % were kept. Next, CD8 T cells were filtered as those expressing *Cd2*, *Cd8a* and *CD8b1* (≥ 1 UMI) but not *Cd4* (0 UMI). Cells expressing *Cd14*, *Csf1r*, *Cd19*, *Spi1*, *Foxp3*, *H2-Aa*, and *H2-Ab1* were further removed, and 1788 high-quality CD8 TIL transcriptomes were obtained.

For dimensionality reduction, highly variable genes (HVG) were first identified using Seurat 3.1.1 *vst* method with default parameters (Stuart *et al.*, Cell, vol. 177, issue 7, p1888-1902.e21, June 13, 2019). Next, mitochondrial, ribosomal protein-coding genes and cell cycle genes (those bearing Gene Ontology term GO:0007049) were removed from the set of HVG, and the remaining HVG (1649) was scaled to have mean=0 and variance=1. Standardized HVG was used for a first step of dimensionality reduction using PCA, and a second set using UMAP (as implemented in Seurat v3.1.1) on the first 10 principal components (with other parameters by default). Clustering was performed using the shared nearest neighbor method of Seurat with parameters using FindNeighbors with default parameters and FindClusters with resolution=0.2.

For supervised classification of CD8 TIL states, TILPRED

(<https://github.com/carmonalab/TILPRED>; Santiago J. Carmona, *et al.*, OncoImmunology, 9:1(2020)) was used with default parameters. Differentially expressed genes between clusters were identified using FindAllMarkers and MAST v1.10 (Finak, G., *et al.* Genome Biol 16, 278 (2015)) with parameters min.pct=0.25 and logfc.threshold=0.25. For comparison of Gzmc cluster and conventional exhausted cluster, the origin 'exhausted' cluster was sub-clustered by increasing the 'resolution' parameter to 0.3. Differential expression analysis between the refined exhaustion cluster and Gzmc cluster was assessed using FindAllMarkers and MAST v1.10 with parameters min.pct=0.1 and logfc.threshold=0.25. Gene set enrichment analysis of these clusters vs. TOX-KO signature (Scott, A.C., *et al.* Nature 571, 270–274 (2019).) was calculated using GSEA function from clusterProfiler package v3.12 (Guangchuang Yu, *et al.*, OMICS: A Journal of Integrative Biology. May 2012.284-287) with default parameters and using the top 200 differentially expressed cluster genes with p-value < 0.01 ordered by decreasing fold-change.

Statistical analysis

Normal Distribution of data was evaluated using the Shapiro-Will normality test. A two-tailed Student's t-test was used to compare two groups (if normal distribution and homoscedasticity), or a t-test with Welch's correction (if normal distribution but not homoscedasticity), and if data were not normally distributed, the non-parametric Mann-Whitney test was used. For comparing more than two groups, a similar strategy was followed. A Kruskal Wallis Test was used if normal distribution was absent. One-way ANOVA test was used if normal distribution and homoscedasticity, or a Brown-Forsythe and Welch ANOVA test was used in case of normal distribution but not homoscedasticity. Correction for multiple comparisons was done using a Dunn's Test (for Kruskal Wallis Test), a Dunnet Test (for one-way ANOVA test), and a Tukey test (for Brown-Forsythe's test). Survival Analysis was done using a log-rank Mantel-Cox model. The Pearson correlation test was used to calculate the correlation between the number of TCF1+ OT-1 intratumoral CD8 T cells and the total number of tumor-infiltrated OT-1. All these statistical analyses were done with GraphPad Prism 8.0, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001

Statistical analysis of tumor control was performed using the change (%) of tumor volume relative to day 17 after tumor inoculation. The best response (smallest tumor volume) observed for each animal after at least 12 days post-1st ACT was taken for the calculation. The Objective

Response rate and Clinical Benefit rate by treatment group were calculated over the total number of mice per group, as (1) Objective Response includes Complete Response (CR; 100% reduction in tumor volume) and Partial Response (PR; $\leq -30\%$ tumor change); and (2) Clinical Benefit includes CR, PR and Stable Disease ($-30\% < \text{tumor change} \leq +20\%$).

Predicted probabilities of the variables “Objective response” and “Clinical benefit” were calculated using exact logistic regression. The values of tumor change as a continuous variable were further analyzed using linear regression. P-values lower than 0.05 were considered as statistically significant.

EXAMPLE 2

Molecular Modeling of PD1/PD-L1

At the time the modeling was performed, no experimental 3D structure was available for the human PD1/PD-L1 complex. Consequently, a structural model was created of the human complex by homology modeling, with the MODELLER program [PMID: 25199792], using the experimental structure of the human PD1 in complex with the mouse PD-L1 (PDB ID 3BIK [PMID: 18287011]), based on the alignment of the human and mouse PD1 sequences. 500 models were generated and ranked according to the MODELLER objective function. The top-ranked model was used to perform the Molecular Dynamics (MD) simulations and binding free energy calculations described below.

Visual inspection of the interface between PD1 and PD-L1 at the atomic level revealed that the 24 following PD1 residues could be potentially subjected to mutation in order to increase its affinity for PD-L1: Val64, Asn66, Tyr68, Met70, Ser73, Asn74, Gln75, Thr76, Asp77, Lys78, Ala81, Pro89, Gly90, Cys123, Leu122, Gly124, Ile126, Leu128, Pro130, Lys131, Ala132, Gln133, Ile134, Glu136. Since several mutations could be envisioned for some of these residues, a total of 55 sequence mutations were tested virtually using a computer-aided approach

The binding free energy change for each amino acid replacement, $\Delta\Delta G_{bind}^{res}$, was calculated using the MM-GBSA approach as the difference between its contributions for the mutated complex, $\Delta G_{bind}^{res,mut}$, and that for the wild type complex, ΔG_{bind}^{res} : $\Delta\Delta G_{bind}^{res} = \Delta G_{bind}^{res,mut} - \Delta G_{bind}^{res}$. The GB-MV2 generalized Born model was used to simulate implicitly the effect of the solvent.

ΔG_{bind}^{res} and $\Delta G_{bind}^{res,mut}$ values were calculated from stochastic boundary conditions MD simulations of the wild type and the corresponding mutated PD1/PDL1 complexes, respectively. 5 MD simulations, each 500 ps in production length, were performed for each of the wild type residues mentioned above. For the mutated proteins, the modified side chain was introduced virtually in the homology model. With the exception of mutations to Ala, several rotamers were taken into account during this phase for the mutated residue, and introduced into the initial model, creating this way several new models (one per rotamer). Again, 5 MD simulations, 500 ps in length, were performed for each rotamer of each mutant. In total, 120 MD simulations were performed on the wild-type system, and 1165 MD simulations were performed for the mutated systems. Each MD simulation was performed after hydrating the above-described homology model (wild-type or virtually mutated) using a 25 Å radius water sphere centered on the C α atom of the above-mentioned residues. Binding free energy terms were calculated as an average of over 100 frames regularly extracted from the 500 ps production trajectory of each MD simulation. The binding free energy difference for a given mutation, $\Delta\Delta G_{bind}^{res}$, was then obtained by summing the $\Delta\Delta G_{bind}^{res}$ values for the mutated residue and all the first-shell residues in contact with it. The final value of $\Delta\Delta G_{bind}^{res}$ was selected as the most favorable value (*i.e.*, the most negative) of all $\Delta\Delta G_{bind}^{res}$ values calculated over all MD simulations relative to that mutation (*i.e.*, from the 5 MD simulation of the wild-type system and from the 5 MD simulations of each of the retained rotamers of the mutated system).

Table 1. Calculated binding free energy change, $\Delta\Delta G_{bind}^{res}$, for the virtual sequence modifications to PD1 decoy in kcal/mol. Mutations are ranked, from the most favorable ones to the binding (*i.e.*, the most negative $\Delta\Delta G_{bind}^{res}$ values) to the least favorable ones.

Mutation	$\Delta\Delta G_{bind}^{res}$	Mutation	$\Delta\Delta G_{bind}^{res}$	Mutation	$\Delta\Delta G_{bind}^{res}$	Mutation	$\Delta\Delta G_{bind}^{res}$
L122E	-19.44	G124S	-3.01	V64I	-0.64	S73Y	5.08
T76D	-14.74	G90I	-2.92	L128Q	-0.50	S73Q	5.43
Q133K	-14.41	G124T	-2.89	A132T	-0.47	Y68K	10.41
T76E	-13.81	L122Q	-2.83	D77E	-0.46	Y68N	15.57
A132W	-12.85	G90V	-2.46	I134L	-0.24	Y68F	17.29
M70E	-11.83	K131Q	-2.35	N66Q	-0.12	E136F	32.31
M70D	-10.8	A132I	-2.18	V64M	-0.11	S73Y	5.08
K78R	-9.28	Q75R	-2.18	V64L	0.01	S73Q	5.43
A132Y	-8.86	Q75K	-2.17	I134M	0.11	Y68K	10.41
A132L	-7.37	I134F	-1.85	Q75L	1.34	Y68N	15.57
A132F	-7.20	S73R	-1.79	Q75I	1.55	Y68F	17.29

Q133R	-6.92	M70L	-1.75	N74E	2.60	E136F	32.31
N74Q	-5.02	G124F	-1.41	T76V	3.30		
S73K	-3.82	G124A	-1.32	T76I	3.91		
G90L	-3.08	A132V	-1.09	T76L	4.07		
K131Y	-3.08	N74D	-0.87	T76F	4.91		

EXAMPLE 3

CD40L Decoy and IL-2 variant and combination GEEP therapy

SIN retroviral vectors were constructed encoding a trimeric CD40L decoy as well as a variant of IL-2 that does not engage CD25. These molecules are expressed under NFAT and hence only produced in an activated T cell, which should only take place in the tumor microenvironment. It was shown in preclinical models that the IL-2 variant promotes a less differentiated phenotype and supports *in vivo* engraftment (*i.e.*, persistence of the T cells). In the preclinical studies, it was also shown that CD40L promotes tumor control. It can act on antigen-presenting cells such as dendritic cells to activate them and thereby provide better T-cell support. Hence, CD40L decoy is a tumor microenvironment re-programmer.

T cells were first engineered with PD1 decoy-tEGFR and then combined, either by co-transduction or by mixing different engineered T cell populations, with the CD40L decoy and IL2^V. The tEGFR (or referred to as Cellular Elimination Tag (CET)) can be used as a means of evaluating transduction efficiency and for enriching the engineered cells (on anti-EGFR coated beads) if necessary. It can be used as a means of tracking the engineered T cells in a patient post-engraftment (via FACS from drawn blood samples or tumor biopsies). In addition, it can be used as an elimination tag via ADCC in the event of toxicity in a patient with Cetuximab.

Table 2. Representative sequences of example transgenes

SEQ ID NO	SEQUENCE	NOTE	Affinity to PD-L1	Solubility
1	SGWLLEVPNGPWRS�TFYPAWLTVSEGANATFTCSLSNWSEDLMLNWNRLSPSNQTEKQAAF SNGLSQPVDARFQIIQLPNRHDFHNMILDTR RNDSGIYLCGAISLHPKAKIEESPGAELVVTER ILETSTRYPSPSPKPEGRFQ	N-terminal ectodomain of murine PD-1 (Uniprot ID: Q02242 residues S21-Q167, C83S)		
2	MQIPQAPWPVWVAVLQLGWRPGWFLDSPDR PWNPPTFSPALLVVTEGDNATFTCSFSNTSESVLNWYRMSPSNQTDKLAAPEDRSQPGQDC RFRVTQLPNGRDFHMSVVRARRNDSTGYLC GAISLAPKAQIKESLRAELRVTERRAEVPTAH PPSPRPAGQFQTLVVGVGGLLGSLLVW VLAVICSRAARGTIGARRTGQPLKEDPSAVPV FSVDYGELDFQWREKTPEPPVPCVPEQTEYA TIVFPSGMGTSSPARRGSADGPRSAQPLRPED GHCSWPL	Human PD1; UniProtKB/Swiss-Prot: Q15116 (PDCD1_HUMAN)		
3	PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESVLNWYRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSTGYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPPSPRPAGQFQ	Human PD1 motif (21-167)		
4	PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESVLNWYRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSTGYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPPSPRPAGQFQPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSPGK	Human PD1.IgG4		

5	CCTGGCTGGTTTCTGGACAGCCCTGACAGA CCCTGGAATCCTCCAACATTTCAGCCCCGCT CTGCTGGTGGTTACCGAGGGCGATAATGCC ACCTTCACCTGTAGCTTCAGCAACACCAGC GAGAGCTTCGTGCTGAACTGGTACAGAATG AGCCCCAGCAACCAGACCGACAAGCTGGCC GCCTTTCCTGAGGATAGATCTCAGCCCCGC CAGGACTGCCGGTTCAGAGTTACACAGCTG CCCAACGGCCGGGACTTCCACATGTCTGTC GTCCGGGCCAGAAAGAAACGACAGCGGCAC ATATCTGTGCGGCGCCATTTCTCTGGCCCCCT AAGGCTCAGATCAAAGAGAGCCTGAGAGC CGAGCTGAGAGTGACAGAAAGACGGGCCG AAGTGCCACAGCTCACCTTCACCTTCTCC AAGACCTGCCGGCCAGTTTCAGCCTCCTTG TCCTAGCTGTCCTGCTCCTGAGTTTCTCGGC GGACCCTCCGTGTTCTGTTTCTCCAAAGC CTAAGGACACCCTGATGATCAGCAGAACCC CTGAAGTGACCTGCGTGGTGGTGGACGTGT CCCAAGAGGACCCTGAGGTGCAGTTCAATT GGTACGTGGACGGCGTGGAAGTGACAAC GCCAAGACCAAGCCTAGAGAGGAACAGTT CAACAGCACCTACAGAGTGGTGTCCGTGCT GACCGTGCTGCACCAGGATTGGCTGAACGG CAAAGAGTACAAGTGCAAGGTGTCCAACA AGGGCCTGCCTAGCAGCATCGAGAAAACCA TCAGCAAGGCCAAGGGCCAGCCAAGAGAA CCCCAGGTGTACACACTGCCTCCAAGCCAA GAGGAAATGACCAAGAACCAGGTGTCCCTG ACCTGCCTGGTCAAGGGCTTCTACCCTTCCG ATATCGCCGTGGAATGGGAGAGCAATGGCC AGCCTGAGAACAACACTACAAGACCACACCTC CTGTGCTGGACAGCGACGGCTCATTCTTCCT GTACAGCAGACTGACCGTGGACAAGAGCA GATGGCAAGAGGGCAACGTGTTTCAGCTGCA GCGTGATGCACGAGGCCCTGCACAACCACT ACACCCAGAAGTCCCTGAGCCTGTCTCCTG GCAA	Human PD1.IgG4		
6	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQDARFRVTQLPNGRDFHMSVVRAR RNDSTYLCAISLAPKAQIKESLRAELRVTE RRALE	WT PD1 (the PD1 variants are based on this sequence as a template below)	Set at 1.0 as a referen ce value	100.0 %
7	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE			

	DRSQPGQDARFRVTQLPNGRDFHMSVVRAR RNDSTYLCGAISLAPKAQIKESLRAELRVTE RRALEHHHHHH			
8	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWyRMSPSNQTDKLAAPFE DRSQPGQDARFRVTQLPNGRDFHMSVVRAR RNDSTYLCGAISLAPKAQIKESLRAELRVTE RRAEVPTAHPSPSPRPAGQFQTPPCPSCPAPE FLGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSQEDPEVQFNWYVDGVEVHNAKTKPR EEQFNSTYRVVSVLTVLHQDWLNGKEYKC KVSNGKLPSSIEKTISKAKGQPREPQVYTLPP SQEEMTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSRLTV DKSRWQEGNVFSCSVMHEALHNHYTQKSLS LSLGK			
9	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWyRESNQQDDRLAAFPED RSQPGQDARFRVTQLPNGRDFHMSVVRARR NDSTYLCGAISLAPKWKIKESLRAELRVTER RALE	Core_W	1018.5	0.1%
10	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWyRESNQQDDRLAAFPED RSQPGQDARFRVTQLPNGRDFHMSVVRARR NDSTYLCGAISLAPKWKIKESLRAELRVTER RALEHHHHHH			
11	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWyRESNQQDDRLAAFPED RSQPGQDARFRVTQLPNGRDFHMSVVRARR NDSTYLCGAISLAPKWKIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEF LGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSQEDPEVQFNWYVDGVEVHNAKTKPRE EQFNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKGLPSSIEKTISKAKGQPREPQVYTLPP SQEEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSRLTV KSRWQEGNVFSCSVMHEALHNHYTQKSLS LSLGK			
12	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWyRESNQQDDRLAAFPED RSQPGQDARFRVTQLPNGRDFHMSVVRARR NDSTYLCGAISLAPKAKIKESLRAELRVTER RALE	Core A	7.1	0.2%

13	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWyRESpsNQ DD RLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY E CGAISLAPKA K IKESLRAELRVTER RALEHHHHHH			
14	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWyRESpsNQ DD RLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY E CGAISLAPKA K IKESLRAELRVTER RALEAEVPTAHPSPPRAGQFQTPPCSCPAP EFLGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSQEDPEVQFNWYVDGVEVHNAKTKPREE QFNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKGLPSSIEKTISKAKGQPREPQVYTLPPSQEE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPE NNYKTPPVLDSDGSFFLYSRLTVDKSRWQE GNVFSCSVMHEALHNHYTQKSLSLGLK			
15	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWyRESpsNQ DD RLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY E CGAISLAPK L IKESLRAELRVTER RALE	Core L	1.7	0.2%
16	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWyRESpsNQ DD RLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY E CGAISLAPK L IKESLRAELRVTER RALEHHHHHH			
17	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWyRESpsNQ DD RLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY E CGAISLAPK L IKESLRAELRVTER RAEVPTAHPSPPRAGQFQTPPCSCPAP EFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNKG LPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTPPVLDSDGSFFLYSRLTVDKSRWQEGNV FSCSVMHEALHNHYTQKSLSLGLK			
18	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWyRESpsNQTDKLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY L CGAISLAPKAQIKESLRAELRVTER RALE	M70E	5.2	87.0 %
19	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWyRESpsNQTDKLAAFPED			

	RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSGTYLCGAISLAPKAQIKESLRAELRVTER RALEHHHHHH			
20	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRESNQTDKLAAPED RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSGTYLCGAISLAPKAQIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQTPPCSPCAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNKG LPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTPPVLDSDGSFFLYSRLTVDKSRWQEGNV FSCSVMHEALHNHYTQKSLSLGLGK			
21	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRMSPSNQDDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCGAISLAPKAQIKESLRAELRVTE RRALE	T_76D	8.3	11.0 %
22	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRMSPSNQDDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCGAISLAPKAQIKESLRAELRVTE RRALEHHHHHH			
23	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRMSPSNQDDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCGAISLAPKAQIKESLRAELRVTE RRAEVPTAHPSPSPRPAGQFQTPPCSPCAPEF LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SQEDPEVQFNWYVDGVEVHNAKTKPREEQF NSTYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTPPVLDSDGSFFLYSRLTVDKSRWQEGN VFSCSVMHEALHNHYTQKSLSLGLGK			
24	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRMSPSNQTDRLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCGAISLAPKAQIKESLRAELRVTE RRALE	K_78R	1.4	56.0 %
25	MASDSPDRPWNPPPTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRMSPSNQTDRLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR			

	RNDSTYLTCGAILAPKAQIKESLRAELRVTE RALEHHHHHH			
26	MASDSPDRPWNPTTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRMSPSNQTDRLAAFPED DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSTYLTCGAILAPKAQIKESLRAELRVTE RAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEF LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SQEDPEVQFNWYVDGVEVHNAKTKPREEQF NSTYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTPPVLDSDGSFFLYSRLTVDKSRWQEGN VFSCSVMHEALHNHYTQKSLSLGLGK			
27	MASDSPDRPWNPTTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRESNQQDDRLAAFPED RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSTYLTCGAILAPKAKIKESLRAELRVTER RALE	X1 4XMut	5.6	12.5 %
28	MASDSPDRPWNPTTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRESNQQDDRLAAFPED RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSTYLTCGAILAPKAKIKESLRAELRVTER RALEHHHHHH			
29	MASDSPDRPWNPTTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRESNQQDDRLAAFPED RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSTYLTCGAILAPKAKIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNKG LPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTPPVLDSDGSFFLYSRLTVDKSRWQEGNV FSCSVMHEALHNHYTQKSLSLGLGK			
30	MASDSPDRPWNPTTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRDSPSNQQDDRLAAFPED RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSTYLTCGAILAPKAKIKESLRAELRVTER RALE	B2 4XB	7.3	0.1%
31	MASDSPDRPWNPTTFSPALLVVTEGDNATFT CSFSNTSEFVLNWYRDSPSNQQDDRLAAFPED RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSTYLTCGAILAPKAKIKESLRAELRVTER RALEHHHHHH			

32	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRDSPSNQ DD RLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTYLCGAISLAPKAKIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNKG LPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTPPVLDSDGSFFLYSRLTVDKSRWQEGNV FSCSVMHEALHNHYTQKSLSLGLGK			
33	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQ DD RLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY I CGAISLAPK W KIKESLRAELRVTER RALE	6XB	1.3	0.0%
34	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQ DD RLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY I CGAISLAPK W KIKESLRAELRVTER RALEHHHHHH			
35	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQ DD RLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY I CGAISLAPK W KIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNKG LPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTPPVLDSDGSFFLYSRLTVDKSRWQEGNV FSCSVMHEALHNHYTQKSLSLGLGK			
36	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQ V DRLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY I CGAISLAPK W KIKESLRAELRVTER RALE	6XC	1.1	0.6%
37	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQ V DRLAAFPED RSQPGQDA <u>R</u> FRVTQLPNGRDFHMSVVRARR NDSGTY I CGAISLAPK W KIKESLRAELRVTER RALEHHHHHH			

38	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQVDRLAAFPED RSQPGQDA ~~R~~ FRVTQLPNGRDFHMSVVRARR NDSGTYICGAISLAPKWKIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNKG LPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTPPVLDSDGSFFLYSRLTVDKSRWQEGNV FSCSVMHEALHNHYTQKSLSLGLGK			
39	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQVDRLAAFPED RSQPGQDA ~~R~~ FRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKAKIKESLRAELRVTER RALE	5XD	1.1	90.0 %
40	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQVDRLAAFPED RSQPGQDA ~~R~~ FRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKAKIKESLRAELRVTER RALEHHHHHH			
41	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQVDRLAAFPED RSQPGQDA ~~R~~ FRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKAKIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNKG LPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTPPVLDSDGSFFLYSRLTVDKSRWQEGNV FSCSVMHEALHNHYTQKSLSLGLGK			
42	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQDA ~~R~~ FRVTQLPNGRDFHMSVVRAR RNDSGTYECGAISLAPKAQIKESLRAELRVTE RALE	L122E	3	2.0%
43	MASDSPDRPWNPPTFSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQDA ~~R~~ FRVTQLPNGRDFHMSVVRAR RNDSGTYECGAISLAPKAQIKESLRAELRVTE RALEHHHHHH			

44	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYECGAISLAPKAQIKESLRAELRVTE RRAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEF LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SQEDPEVQFNWYVDGVEVHNAKTKPREEQF NSTYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTTPVLDSGDSFFLYSRLTVDKSRWQEGN VFSCSVMHEALHNHYTQKSLSLGLGK			
45	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCCGAISLAPKWQIKESLRAELRVTE RRALE	A132W	3	41.0 %
46	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCCGAISLAPKWQIKESLRAELRVTE RRALEHHHHHH			
47	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCCGAISLAPKWQIKESLRAELRVTE RRAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEF LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SQEDPEVQFNWYVDGVEVHNAKTKPREEQF NSTYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTTPVLDSGDSFFLYSRLTVDKSRWQEGN VFSCSVMHEALHNHYTQKSLSLGLGK			
48	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCCGAISLAPKAKIKESLRAELRVTE RRALE	Q133K	3	100.0 %
49	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCCGAISLAPKAKIKESLRAELRVTE RRALEHHHHHH			

50	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQTDKLAAPFE DRSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRAR RNDSGTYLCGAISLAPKAKIKESLRAELRVTE RRAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEF LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SQEDPEVQFNWYVDGVEVHNAKTKPREEQF NSTYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEEMT KNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTTPVLDSGDSFFLYSRLTVDKSRWQEGN VFSCSVMHEALHNHYTQKSLSLGLGK			
51	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESNQNVDRLAAFPED RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKWKIKESLRAELRVTER RALE	6xMUT	ND	0.1%
52	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESNQNVDRLAAFPED RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKWKIKESLRAELRVTER RALEHHHHHH			
53	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESNQNVDRLAAFPED RSQPGQD <u>A</u> RFRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKWKIKESLRAELRVTER RAEVPTAHPSPSPRPAGQFQTPPCPSCPAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNKG LPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSGDSFFLYSRLTVDKSRWQEGNV FSCSVMHEALHNHYTQKSLSLGLGK			
54	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQDDRLAAFPED DRSQPGQDARFRVTQLPNGRDFHMSVVRAR RNDSGTYFCGAISLAPKAKIKESLRAELRVTE RALE	PD1_4XMU T_M70	10	50.0 %
55	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQDDRLAAFPED DRSQPGQDARFRVTQLPNGRDFHMSVVRAR RNDSGTYFCGAISLAPKAKIKESLRAELRVTE RALEHHHHHH	PD1_4XMU T_M70 (with 6xHis)		

56	DSPDRPWNPPFTSPALLVVTEGDNATFTCSFS NTSESFVLNWYRMSPSNQDDRLAAFPEDRSQ PGQDARFRVTQLPNGRDFHMSVVRARRNDS GTYFCGAISLAPKAKIKESLRAELRVTERRAE <u>VPTAHPSPPRPAQGFQTPPCPSCPAPEFLGG</u> PSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEE MTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSGSSFLYSRLTVDKSR WQEGNVFSCSVMHEALHNHYTQKSLSL GK	PD1_4XMU T_M70/IgG4 Fc IgG4Fc is bolded and spacing residues are underlined		
57	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQVDRLAAFP EDRSQPGQDARFRVTQLPNGRDFHMSVVRAR RNDSTYFCGAISLAPKWKIKESLRAELRVTE RRALE	PD1_6XDM		
58	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRMSPSNQVDRLAAFP EDRSQPGQDARFRVTQLPNGRDFHMSVVRAR RNDSTYFCGAISLAPKWKIKESLRAELRVTE RRALEHHHHHH	PD1_6XDM (with 6xHis)	7.5	38.0 %
59	DSPDRPWNPPFTSPALLVVTEGDNATFTCSFS NTSESFVLNWYRMSPSNQVDRLAAFPEDRSQ PGQDARFRVTQLPNGRDFHMSVVRARRNDS GTYFCGAISLAPKWKIKESLRAELRVTERRAE <u>VPTAHPSPPRPAQGFQTPPCPSCPAPEFLGG</u> PSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEE MTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSGSSFLYSRLTVDKSR WQEGNVFSCSVMHEALHNHYTQKSLSL GK	PD1_6XDM/ IgG4Fc IgG4Fc is bolded and spacing residues are underlined		
60	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQDDRLAAFP EDRSQPGQDARFRVTQLPNGRDFHMSVVRAR RNDSTYFCGAISLAPKAKIKESLRAELRVTER RALE	PD1_4XMU T		
61	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESFVLNWYRESPSNQDDRLAAFP EDRSQPGQDARFRVTQLPNGRDFHMSVVRAR RNDSTYFCGAISLAPKAKIKESLRAELRVTER RALEHHHHHH	PD1_4XMU T (with 6xHis)	5	59.0 %

62	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESVFLNHWYRESNQNDDRLAAFPED RSQPGQDARFRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKAKIKESLRAELRVTER <u>RAEVPTAHPSPPRPAGQFQTPPCPSCPAPEF</u> LGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSQEDPEVQFNWYVDGVEVHNAKTKPRE EQFNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKGLPSSIEKTISKAKGQPREPQVYTLPP SQEEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSRLTVD KSRWQEGNVFSCSVMEALHNHYTQKSLS LSLGK	PD1_4XMU T/IgG4Fc IgG4Fc is bolded and spacing residues are underlined		
63	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESVFLNHWYRESNQNVDRLAAFPED RSQPGQDARFRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKWKIKESLRAELRVTER RALE	PD1_6XD		
64	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESVFLNHWYRESNQNVDRLAAFPED RSQPGQDARFRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKWKIKESLRAELRVTER RALEHHHHHH	PD1_6XD (with 6xHis)	7.2	38.0 %
65	MASDSPDRPWNPPFTSPALLVVTEGDNATFT CSFSNTSESVFLNHWYRESNQNVDRLAAFPED RSQPGQDARFRVTQLPNGRDFHMSVVRARR NDSGTYFCGAISLAPKWKIKESLRAELRVTER <u>RAEVPTAHPSPPRPAGQFQTPPCPSCPAPEF</u> LGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSQEDPEVQFNWYVDGVEVHNAKTKPRE EQFNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKGLPSSIEKTISKAKGQPREPQVYTLPP SQEEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSRLTVD KSRWQEGNVFSCSVMEALHNHYTQKSLS LSLGK	PD1_6XD/Ig G4Fc IgG4Fc is bolded and spacing residues are underlined		
66	PPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSQEDPEVQFNWYVDGVEVHNA KTKPREEQFNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKGLPSSIEKTISKAKGQPREPQVYT LPPSQEEMTKNQVSLTCLVKGFYPSDIAVEW ESNGQPENNYKTTPPVLDSDGSFFLYSRLTVD KSRWQEGNVFSCSVMEALHNHYTQKSLSLS PGK	Human IgG4 motif (104- 327)		

67	ASTKGPSVFPLAPCSRSTSESTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQYTCNVDPKPSNTKVD KRVESKYGGPPCPSCPAPEFLGGPSVFLFPPKPK DTLMISRTPEVTCVVDVDSQEDPEVQFNWYV DGVEVHNAKTKPREEQFNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKGLPSSIEKTISKAKG QPREPQVYTLPPSQEEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSDGSF FLYSRLTVDKSRWQEGNVFSCSVMHEALHN HYTQKSLSLGK	human IgG4_Fc (Uniprot ID: P01861.1)		
68	DVQMIQSPSSLSASLGDIVTMTQCASQGTSIN LNWFQKPGKAPKLLIYGASILEDGVPSRFSG SRYGTDFTLTISSEDEDMATYFCLQHSYLPY TFGGGTKLEIKR	murine IgG Kappa Chain region V-III MOPC 321; Uniprot ID: P01650		
69	MYRMQLLSIALSLALVTNSAPTSSTKKTQL QLEHLLLDLQMLNGINNYKNPKLTRMLTFK FYMPKKATELKHLQCLEEELKPLEEVLNLAQ SKNFHLRPRDLISNINVIVLELKGSETTFMCEY ADETATIVEFLNRWITFCQSIISTLT	Human Interleukin 2 (IL2); UniProtKB/S wiss-Prot: P60568 (IL2_HUMAN)		
70	APTSSSTKKTQLQLEHLLLDLQMLNGINNYK NPKLTRMLTFKFYMPKKATELKHLQCLEEEL KPLEEVLNLAQSKNFHLRPRDLISNINVIVLEL KGSETTFMCEYADETATIVEFLNRWITFCQSII STLT	Truncated IL-2		
71	APTSSSTKKTQLQLEHLLLDLQMLNGINNYK NPKLTAMLTAKFAMPKKATELKHLQCLEEAL KPLEEVLNLAQSKNFHLRPRDLISNINVIVLEL KGSETTFMCEYADETATIVEFLNRWITFSQSII STLT	IL2V (R38A F42A Y45A E62A, C145S)		
72	GCCCCTACCAGCAGCAGCACCAAAAAGAC ACAGCTGCAACTGGAACACCTCCTGCTGGA CCTGCAGATGATCCTGAACGGCATCAACAA CTACAAGAACCCCAAGCTGACCGCCATGCT GACAGCCAAGTTCGCCATGCCTAAGAAGGC CACCGAGCTGAAGCACCTCCAGTGCCTGGA AGAGGCCCTGAAGCCTCTGGAAGAAGTGCT GAATCTGGCCCAGAGCAAGAACTTCCACCT GAGGCCTAGGGACCTGATCAGCAACATCAA CGTGATCGTGCTGGAAGTGAAGGGCAGCGA GACAACCTTCATGTGCGAGTACGCCGACGA	IL2V (R38A F42A Y45A E62A); cDNA		

	GACAGCTACCATCGTGGAATTTCTGAACCG GTGGATCACCTTCAGCCAGAGCATCATCAG CACCTGACCTGA			
73	SITGISPITEYLASLSTYNDQSITFALEDESIEIY VEDLKKDEKKDKVLLSYYESQHPSNESGDGV DGKMLMVTLSPTKDFWLHANNKEHSVELHK CEKPLPDQAFFVLHNMHSNCVSFECKTDPGV FIGVKDNHLALIKVDSSSENLC TENILFKLSET	Human Interleukin 33 (IL-33) motif (112- 270)		
74	AGCATCACCGGCATCAGCCCCATCACAGAG TATCTGGCCAGCCTGAGCACCTACAACGAC CAGAGCATCACATTCGCCCTGGAAGATGAG AGCTACGAGATCTACGTGGAAGATCTGAAG AAGGACGAGAAGAAAGACAAGGTGCTGCT GAGCTACTACGAGTCTCAGCACCCAGCAA TGAGTCTGGCGACGGCGTGGACGGAAAGAT GCTGATGGTTACTGAGCCCCACCAAGGA TTTCTGGCTGCACGCCAACAACAAGAGCA CAGCGTCGAGCTGCACAAGTGCGAGAAGCC TCTGCCTGACCAGGCCTTCTTCGTGCTGCAC AACATGCACAGCAACTGCGTGTCCTTCGAG TGCAAGACCGATCCTGGCGTGTCATCGGC GTGAAGGACAACCATCTGGCCCTGATCAAG GTGGACAGCAGCGAGAATCTGTGCACCGAG AACATCCTGTTCAAGCTGAGCGAGACA	Human IL-33 motif		
75	SIQGTSLLTQSPASLSTYNDQSVSFVLENGCY VINVDDSGKDQEQDQVLLRYYESPCPASQSG DGVDGKKLMVMNMSPIKDTDIWLHANDKDYS VELQRGDVSPPEQAFFVLHKKSSDFVSFECKN LPGTYIGVKDNQLALVEEKDESCNNIMFKLS KI	murine IL-33 (Uniprot ID:Q8BVZ5. 1, residues S109-I266)		
76	MEESVVRPSVFVVDGQTDIPFTRLGRSHRRQS CSVARVGLGLLLLLMGAGLAVQGWFLQLH WRLGEMVTRLPDGPAGSWEQLIQERRSHEVN PAAHLTGANSSLTGSGGPLLWETQLGLAFLR GLSYHDGALVVTKAGYYYIYSKVQLGGVGC PLGLASTITHGLYKRTPRYPEELELLVSQQSPC GRATSSSRVWWDSSFLGGVVHLEAGEKVVV RVLDERLVRLRDGTRSYFGAFMV	Human LIGHT (LIGHT); UniProtKB/S wiss-Port: O43557 (TNF14_HU MAN)		
77	DGPAGSWEQLIQERRSHEVNPA AHLTGANSS LTGSGGPLLWETQLGLAFLRGLSYHDGALVV TKAGYYYIYSKVQLGGVGCPLGLASTITHGL YKRTPRYPEELELLVSQQSPCGRATSSSRVW WDSSFLGGVVHLEAGEKVVVRLDERLVRL RDGTRSYFGAFMV	Human LIGHT motif (74-240)		

78	GATGGACCTGCCGGATCTTGGGAGCAGCTG ATCCAAGAGCGGAGAAGCCACGAAGTGAA CCCTGCCGCACATCTGACAGGCGCCAATTC TAGCCTGACAGGCTCTGGTGGACCCCTGCT GTGGGAAACTCAACTGGGACTCGCCTTCCT GAGAGGCCTGAGCTATCATGATGGCGCCCT GGTGGTTACCAAGGCCGGCTACTACTACAT CTACAGCAAGGTGCAGCTCGGCGGCGTGGG ATGTCCTCTTGGACTGGCCTCTACAATCACC CACGGCCTGTACAAGCGGACCCCTAGATAC CCCGAGGAACTGGAAGTCTGGTGTCCCAG CAGAGCCCTTGTGGCAGAGCCACAAGCAGC AGCAGAGTTTGGTGGGACAGCAGCTTTCTC GGCGGAGTGGTGCATCTGGAAGCCGGCGA AAAGGTGGTCGTCAGAGTGTGGATGAGAG ACTCGTGCGGCTGAGAGATGGCACCAGAAG CTACTTCGGCGCCTTCATGGTT	Human LIGHT motif; cDNA		
79	DGGKGSWEKLIQDQRSHQANPAAHLTGANA SLIGIGGPLLWETRLGLAFLRGLTYHDGALVT MEPGYYYVYSKVQLSGVGCPQGLANGLPITH GLYKRTSRYPKELELLVSRSPCGRANSSRV WWDSSFLGGVVHLEAGEEVVVRVPGNRLVR PRDGTRSYFGAFMV	murine LIGHT (Uniprot ID:Q9QYH9. 1, residues D72-V239)		
80	MIETYNQTSRPSAATGLPISMKIFMYLLTVFLI TQMIGSALFAVYLHRRLDKIEDERNLHEDFVF MKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDI MLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGK QLTVKRQGLYYIYAQVTFCNREASSQAPFIA SLCLKSPGRFERILLRAANTHSSAKPCGQQSIH LGGVFELQPGASVFNVTDPQSQVSHGTGFTSF GLLKL	Human CD40 Ligand (CD40L); UniProtKB/S wiss-Port: P29965 (CD40L_HU MAN)		
81	MQRGDEDPQIAAHVVSEANSNAASVLQWAK KGYTMMKSNLVMLENGKQLTVKREGLYYV YTQVTFCNREPSSQRPFIVGLWKPSSGSERI LLKAANTHSSSQLCEQQSVHLGGVFELQAGA SVFVNVTEASQVIHRVGFSSFGLLKL	murine CD40L (Uniprot ID:P27548, residues M112-L260)		
82	MQKGDQNPQIAAHVISEASSKTTSVLQWAEK GYTMSNNLVTLENGKQLTVKRQGLYYIYA QVTFCNREASSQAPFIASLCLKSPGRFERILL RAANTHSSAKPCGQQSIHLGGVFELQPGASV FNVTDPQSQVSHGTGFTSFGLLKL	Human CD40 Ligand motif (113- 261)		

83	ATGCAGAAGGGCGACCAGAATCCTCAGATC GCCGCTCACGTGATCAGCGAGGCCAGCAGC AAGACAACAAGCGTGCTGCAGTGGGCCGA GAAGGGCTACTACACCATGAGCAACAACCT GGTCACCCTGGAAAACGGCAAGCAGCTGAC CGTGAAGAGACAGGGCCTGTACTACATCTA CGCCCAAGTGACCTTCTGCAGCAACAGAGA GGCCAGCTCTCAGGCCCCTTTTATCGCCAG CCTGTGCCTGAAGTCCCCTGGCAGATTCGA GCGGATTCTGCTGAGAGCCGCCAACACACA CAGCAGCGCCAAACCTTGTGGCCAGCAGTC TATTCACCTCGGCGGAGTGTTTGAGCTGCA GCCTGGCGCAAGCGTGTTCTGTGAATGTGAC AGACCCTAGCCAGGTGTCCACGGCACCGG CTTTACATCTTTTCGGCCTGCTGAAGCTG	Human CD40 Ligand motif; cDNA		
84	MGTNKCLLQIALLLCFSTTALSRMKQIEDK IEEILSKIYHIENEIARIKKLIGEVGGGSGGG <u>SGGGSMQKGDQNPQIAAHVISEASSKTTSVL</u> QWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCSNREASSQAPFIASLCLKSPGRF ERILLRAANTHSSAKPCGQQSIHLGGVFELQP GASVFNVTDPQSQVSHGTGFTSFGLLKL	Zipper (bolded)- Linker (underlined)- Extracellular region of CD40L		
85	ATGGGAACAAACAAATGCCTGCTGCAGATC GCCCTGCTGCTGTGCTTTAGCACAAACAGCC CTGAGCCGGATGAAGCAGATCGAGGACAA GATCGAAGAGATCCTGAGCAAGATCTACCA CATCGAGAACGAGATCGCCCGGATCAAGA AACTGATCGGCGAAGTCGGCGGAGGCTCTG GTGGTGGATCTGGCGGAGGATCTATGCAGA AAGGCGACCAGAATCCTCAGATCGCCGCTC ACGTGATCAGCGAGGCCAGCAGCAAGACA ACAAGCGTGCTGCAGTGGGCCGAGAAGGG CTACTACACCATGAGCAACAACCTGGTCAC CCTGGAAAACGGCAAGCAGCTGACCGTGA AGAGACAGGGCCTGTACTACATCTACGCCC AAGTGACCTTCTGCAGCAACAGAGAGGCCA GCTCTCAGGCCCTTTTATCGCCAGCCTGTG CCTGAAGTCCCCTGGCAGATTCGAGCGGAT TCTGCTGAGAGCCGCCAACACACACAGCAG CGCCAAACCTTGTGGCCAGCAGTCTATTCA CCTCGGCGGAGTGTTTGAGCTGCAGCCTGG CGCAAGCGTGTTCTGTGAATGTGACAGACCC TAGCCAGGTGTCCACGGCACCGGCTTTAC ATCTTTTCGGCCTGCTGAAGCTCTGA	Zipper- Linker- Extracellular region of CD40L		
86	MIETYNQTSPPSAATGLPISMKIFMYLLTVFLI TQMIGSALFAVYLHRRLDKIEDERNLHEDFVF	CD40 ligand [Homo		

	MKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDI MLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGK QLTVKRQGLYYIYAQVTFCSNREASSQAPFIA SLCLKSPGRFERILLRAANTHSSAKPCGQQSIH LGGVFELQPGASVFNVTDPVSQVSHGTGFTSF GLLKL	sapiens] Sequence ID: NP_000065. 1		
87	MNNRWILHAAFLLCFSTTALSINYKQLQLQE RTNIRKCCELLEQLNGKINLTYRADFKIPMEM TEKMQKSYTAFAIQEMLQNVFLVFRNNFSST GWNETIVVRLDELHQQT VFLKTVLEEKQEE RLTWEMSSTALHLKSYWVRVQRYLKLMMKY NSYAWMVVRAEIFRNFLIRRLTRNFQN	murine IFN- beta (Uniprot ID:P01575.1)		
88	MLLLVTSLLLCELPHPAFLIPRKVCNGIGIGE FKDSLSINATNIKHFKNCTISISGDLHILPVAFR GDSFTHTPPLDPQELDILKTVKEITGFLLIQAW PENRTDLHAFENLEIIRGRTKQHGQFSLAVVS LNITSLGLRSLKEISDGDVIISGNKNLCYANTI NWKKLFGTSGQKTKIISNRGENSCKATGQVC HALCSPEGCWGPEPRDCVSCRNVSRGRECV KCNLLEGEPRFVENSECIQCHPECLPQAMNI TCTGRGPDNCIQCAHYIDGPHCVKTCAPAGVM GENNTLVWKYADAGHVCHLCHPNCTYGCTG PGLEGCPNTGPKIPSIATGMVGALLLLLVVAL GIGLFM	tEGFR (286 amino acids)		
89	ATGCTTCTGCTGGTTACATCTCTGCTGCTGT GCGAGCTGCCCCATCCTGCCTTTCTGCTGAT CCCCAGAAAAGTGTGCAACGGCATCGGCAT CGGAGAGTTCAAGGACAGCCTGAGCATCAA CGCCACCAACATCAAGCACTTCAAGAACTG CACCAGCATCAGCGGCGACCTGCACATTCT GCCTGTGGCCTTTAGAGGCGACAGCTTCAC CCACACACCTCCACTCGATCCCCAAGAGCT GGACATCCTGAAAACCGTGAAAGAGATCAC CGGATTTCTGTTGATCCAGGCTTGCCCCGA GAACCGGACAGATCTGCACGCCTTCGAGAA CCTGGAAATCATCAGAGGCCGGACCAAGCA GCACGGCCAGTTTTCTCTGGCTGTGGTGTCC CTGAACATCACCAGCCTGGGCCTGAGAAGC CTGAAAGAAATCAGCGACGGCGACGTGATC ATCTCCGGCAACAAGAACCTGTGCTACGCC AACACCATCAACTGGAAGAAGCTGTTCCGGC ACCAGCGGCCAGAAAACAAAGATCATCAG CAACCGGGGCGAGAACAGCTGCAAGGCTA CAGGCCAAGTGTGCCACGCTCTGTGTAGCC CTGAAGGCTGTTGGGGACCCGAGCCTAGAG	tEGFR		

	ATTGCGTGTCTGCAGAAACGTGTCCCGGG GCAGAGAATGCGTGGACAAGTGCAATCTGC TGGAAGGCGAGCCCCGCGAGTTCGTGGAAA ACAGCGAGTGCATCCAGTGTACCCCCGAGT GTCTGCCCCAGGCCATGAACATTACCTGTA CCGGCAGAGGCCCGACAACGTATTTCAGT GCGCCCACTACATCGACGGCCCTCACTGCG TGAAAACATGTCCTGCTGGCGTGATGGGAG AGAACAACACCCTCGTGTGGAAGTATGCCG ACGCCGGACATGTGTGCCACCTGTGTCACC CTAATTGCACCTACGGCTGTACAGGCCCTG GCCTGGAAGGCTGTCCAACAAACGGACCTA AGATCCCCCTCTATCGCCACCGGCATGGTTG GAGCCCTGCTGCTGCTTCTGGTTGTGGCCCT TGGCATCGGCCTGTTTATGTAG			
90	DSPDRPWNPPTFSPALLVVTEGDNATFTCSFS NTSESFVLNWYRMSPSNQDDRLAAFPEDRSQ PGQDARFRVTQLPNGRDFHMSVVRARRNDS GTYFCGAISLAPKAKIKESLRAELRVERRAE VPTAHPSPSPRAGQFQTPPCPSCPAPEFLGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEE MTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTPPVLDSDGSFFLYSRLTVDKSR WQEGNVFSCSVMEALHNHYTQKSLSLSL <u>GKASRKRRSGSGEGRGSLLTCGDVEENPGPM</u> <i>LLLVTSLLLCELPHPAFLIPRKVCNGIGIGEFKD</i> <i>SLSINATNIKHFKNCTISISGDLHILPVAFRGDSFT</i> <i>HTPPLDPQELDILKTVKEITGFLLIQAWPENRTD</i> <i>LHAFENLEIIRGRTKQHGQFSLAVVSLNITSLGLR</i> <i>SLKEISDGDVIISGNKNLCYANTINWKKLFGTSG</i> <i>QKTKIISNRGENSCKATGQVCHALCSPEGCWGP</i> <i>EPRDCVSCRNVSRGRECVDKCNLLEGEPRFVE</i> <i>NSECIQCHPECLPQAMNITCTGRGPDNCIQCAH</i> <i>YIDGPHCVKTCAPAGVMGENNTLVWKYADAGHV</i> <i>CHLCHPNCTYGCTGPGLEGCTNGPKIPSIATG</i> <i>MVGALLLLVVALGIGLFM</i>	PD1_4XMU T_M70_IgG 4Fc/T2A/tE GFR IgG4Fc is bolded; furin cleavage site is double- underlined; T2A is underlined; and tEGFR is italic		

91	GACAGCCCTGACAGACCCTGGAATCCTCCA ACATTCAGCCCCGCTCTGCTGGTGGTTACC GAGGGCGATAATGCCACCTTCACCTGTAGC TTCAGCAACACCAGCGAGAGCTTCGTGCTG AACTGGTACAGAAtGAGCCCCAGCAACCAG GACGACAGACTGGCCGCCTTTCCTGAGGAT AGATCTCAGCCCGGCCAGGACGCCCGGTTC AGAGTTACACAGCTGCCCAACGGCCGGGAC TTCCACATGTCTGTCGTCCGGGCCAGAAGA AACGACAGCGGCACATATTTTTCGGCGCC ATTTCTCTGGCCCCTAAGGCTAAAATCAAA GAGAGCCTGAGAGCCGAGCTGAGAGTGAC AGAAAGACGGGCCGAAGTGCCACAGCTC ACCCTTCACCTTCTCCAAGACCTGCCGGCC AGTTTCAGACTCCTCCTTGTCTAGCTGT CCTGCTCCTGAGTTTCTCGGCGGACCCT CCGTGTTCTGTTTCTCCAAAGCCTAAG GACACCCTGATGATCAGCAGAACCCCTG AAGTGACCTGCGTGGTGGTGGACGTGTC CCAAGAGGACCCTGAGGTGCAGTTCAAT TGGTACGTGGACGGCGTGGAAGTGACA ACGCCAAGACCAAGCCTAGAGAGGAACA GTTCAACAGCACCTACAGAGTGGTGTCC GTGCTGACCGTGCTGCACCAGGATTGGC TGAACGGCAAAGAGTACAAGTGCAAGGT GTCCAACAAGGGCCTGCCTAGCAGCATC GAGAAAACCATCAGCAAGGCCAAGGGCC AGCCAAGAGAACCCAGGTGTACACACT GCCTCCAAGCCAAGAGGAAATGACCAAG AACCAGGTGTCCCTGACCTGCCTGGTCA AGGGCTTCTACCCTTCCGATATCGCCGT GGAATGGGAGAGCAATGGCCAGCCTGAG AACAACTACAAGACCACACCTCCTGTGCT GGACAGCGACGGCTCATTCTTCCTGTAC AGCAGACTGACCGTGGACAAGAGCAGAT GGCAAGAGGGCAACGTGTTCAGCTGCAG CGTGATGCACGAGGCCCTGCACAACCAC TACACCAGAAGTCCCTGAGCCTGTCTCT GGGCAAAGCCAGCCGGAAGAGAAGATCTG GATCTGGCGAAGGCAGAGGCTCCCTGCTGA CTTGCGGAGATGTGGAAGAGAACCCCGGAC CTATGCTTCTGCTGGTTACATCTCTGCTGCTGT GCGAGCTGCCCCATCCTGCCTTCTGCTGATC CCCAGAAAAGTGTGCAACGGCATCGGCATCG GAGAGTTCAAGGACAGCCTGAGCATCAACGCC ACCAACATCAAGCACTTCAAGAACTGCACCAG	PD1_4XMU T_M70_IgG 4Fc/T2A/tE GFR IgG4Fc is bolded; furin cleavage site is double- underlined; T2A is underlined; and tEGFR is italic		
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	<i>CATCAGCGGCGACCTGCACATTCTGCCTGTGG CCTTTAGAGGCGACAGCTTCACCCACACACCT CCACTCGATCCCCAAGAGCTGGACATCCTGAA AACCGTGAAAGAGATCACCGGATTCTGTGGA TCCAGGCTTGGCCCGAGAACCGGACAGATCT GCACGCCTTCGAGAACCTGGAAATCATCAGAG GCCGGACCAAGCAGCACGGCCAGTTTTCTCTG GCTGTGGTGTCCCTGAACATCACCAGCCTGGG CCTGAGAAGCCTGAAAGAAATCAGCGACGGC GACGTGATCATCTCCGGCAACAAGAACCTGTG CTACGCCAACACCATCAACTGGAAGAAGCTGT TCGGCACCAGCGGCCAGAAAACAAAGATCATC AGCAACCGGGGCGAGAACAGCTGCAAGGCTA CAGGCCAAGTGTGCCACGCTCTGTGTAGCCCT GAAGGCTGTTGGGGACCCGAGCCTAGAGATT GCGTGTCTCTGCAGAAACGTGTCCCGGGGCAG AGAATGCGTGGACAAGTGCAATCTGCTGGAAG GCGAGCCCCGCGAGTTCGTGGAAAACAGCGA GTGCATCCAGTGTACCCCGAGTGTCTGCCCC AGGCCATGAACATTACCTGTACCGGCAGAGGC CCCGACAACCTGTATTAGTGCGCCCACTACAT CGACGGCCCTCACTGCGTGAAAACATGTCCTG CTGGCGTGATGGGAGAGAAACAACACCCTCGT GTGGAAGTATGCCGACGCCGGACATGTGTGC CACCTGTGTACCCCTAATTGCACCTACGGCTG TACAGGCCCTGGCCTGGAAGGCTGTCCAACAA ACGGACCTAAGATCCCCTCTATCGCCACCGGC ATGGTTGGAGCCCTGCTGCTGCTTCTGGTTGT GGCCCTTGGCATCGGCCTGTTTATGTAG</i>			
92	DSPDRPWNPTFSPALLVTEGDNATFTCSFS NTSESVLNLWYRMSPSNQVDRLAAFPEDRSQ PGQDARFRVTQLPNGRDFHMSVVRARRNDS GTYFCGAISLAPKWKIKESLRAELRVTERPP CPSCPAPEFLGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSQEDPEVQFNWYVDGVEVH NAKTKPREEQFNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKGLPSSIEKTISKAKGQPR EPQVYTLPPSQEEMTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTPPVLDSDGSF FLYSRLTVDKSRWQEGNVFSCSVMHEALH NHYTQKSLSLSLGKASRKRRSGSGEGRGSL <u>TCGDVEENPGPMLLLVTSLLLCELPHPAFLIPR</u> <u>KVCNGIGIGEFKDSLSINATNIKHFKNCTISISGDL</u> <u>HILPVAFRGDSFTHTPPLDPQELDILKTVKEITG</u> <u>FLLIQAWPENRTDLHAFENLEIRGRKQHGQFS</u> <u>LAVVSLNITSLGLRSLKEISDGDVVISGNKNLCYAN</u>	PD1_6XDM _IgG4Fc/T2 A/tEGFR IgG4Fc is bolded; furin cleavage site is double- underlined; furin cleavage site is double- underlined; T2A is underlined; and tEGFR is italic		

	<i>TINWKKLFGTSGQKTKIISNRGENSCKATGQVC</i> <i>HALCSPEGCWGPEPRDCVSCRNVSRGRECVDK</i> <i>CNLEGEPRFEVENSECIQCHPECLPQAMNITC</i> <i>TGRGPDNCIQCAHYIDGPHCVKTCAPAGVMGEN</i> <i>NTLVWKYADAGHVCHLCHPNCTYGCTGPGLEG</i> <i>CPTNGPKIPSIATGMVGALLLLLVVALGIGLFM</i>			
93	MLLLVTSLLLCELPHPAFLLIPTQVCTGTDMK LRLPASPTHLDMLRHL YQGCQVVQGNLELT YLPTNASLSFLQDIQEVQGYVLIHNPVRQVP LQRLRIVRGTQLFEDNYALAVLDNGDPLNNT TPVTGASPGGLRELQLRSLTEILKGGVLIQRNP QLCYQDTILWKDIFHKNNQLALTLIDTNRSRA CHPCSPMCKGSRGWGESSEDCQSLTRTV CAG GCARCKGPLPTDCCHEQCAAGCTGPKHSDCL ACLHFNHSGICELHCPALVTYNTDTFESMPNP EGRYTFGASCVTACPYNYLSTDVGSCTLVCP LHNQEVTAEDGTQRCEKCSKPCARVCYGLG MEHLREVRAVTSANIQEFAGCKKIFGSLAFLP ESFDGDPASNTAPLQPEQLQVFETLEEITGYL YISAWPDSLPLDSVFQNLQVIRGRILHNGAYS LTLQGLGISWLGLRSLRELGSGLALIHNTHL CFVHTVPWDQLFRNPHQALLHTANRPEDECV GEGlachQLCARGHCWGPPTQCVNCSQFL RGQECVEECRVLQGLPREYVNARHCLPCHPE CQPQNGSVTCFGPEADQCVACAHYKDPPFCV ARCPSGVKPDL SYMPIWKFPDEEGACQPCPIN CTHSCVDLDDKGCPAEQRASPLTSIISAVVGIL LVVVLGVVFGILI	tHER2/ErbB 2 (675 amino acids)		

94	ATGCTTCTGCTGGTTACATCTCTGCTGCTGT GCGAGCTGCCCCATCCTGCCTTTCTGCTGAT CCCCACACAAGTGTGCACCGGCACCGACAT GAAGCTGAGACTGCCTGCCTCTCCTGAGAC ACACCTGGACATGCTGAGACACCTGTACCA GGGCTGTCAGGTGGTGCAGGGCAATCTGGA ACTGACCTACCTGCCTACCAACGCCAGCCT GAGCTTTCTGCAGGACATCCAAGAGGTGCA GGGATACGTGCTGATTGCCACAATCAAGT GCGCCAGGTGCCACTGCAGCGGCTGAGAAT CGTTAGAGGCACCCAGCTGTTGAGAGACAA CTATGCCCTGGCCGTGCTGGACAATGGCGA CCCTCTGAACAACACCACACCTGTGACAGG CGCTTCTCCTGGCGGACTGAGAGAACTGCA GCTGAGAAGCCTGACCGAGATCCTGAAAGG CGGCGTGCTGATCCAGAGAAACCCTCAGCT GTGCTACCAGGACACCATCCTGTGGAAGGA CATCTTCCACAAGAACAACCAGCTGGCCCT GACACTGATCGACACCAACAGAAGCAGAG CCTGCCATCCTTGCAGCCCCATGTGCAAGG GCTCTAGATGTTGGGGCGAGAGCAGCGAGG ATTGCCAGAGCCTGACCAGAACAGTGTGTG CCGGCGGATGTGCCAGATGCAAAGGACCTC TGCTACCGACTGCTGCCACGAGCAATGTG CCGCTGGATGTACAGGCCCAAGCACTCTG ATTGCCTGGCCTGCCTGCACTTCAACCACTC TGGAATCTGCGAACTGCACTGCCCCGCTCT GGTCACCTACAACACCGATACTTCGAGAG CATGCCCAATCCTGAGGGCAGATACACCTT CGGCGCCAGCTGTGTGACAGCCTGTCCTTA CAACTACCTGAGCACCGACGTGGGCAGCTG TACCCTTGTGTGCCCTCTGCATAATCAAGA AGTGACCGCCGAGGACGGCACCCAGAGAT GCGAGAAGTGTAGCAAGCCTTGCGCCAGAG TGTGTTACGGCCTCGGCATGGAACACCTGA GAGAAGTGCGGGCCGTGACCAGCGCCAAT ATCCAAGAATTTGCCGGCTGCAAGAAGATC TTTGGCAGCCTCGCCTTCCTGCCTGAGAGCT TCGATGGCGATCCTGCCAGCAATACTGCC CTCTGCAGCCTGAACAGCTCCAGGTGTTG AGACACTGGAAGAGATCACCGGCTACCTGT ATATCAGCGCCTGGCCAGACAGCCTGCCTG ACCTGTCCGTGTTCCAGAACCTGCAAGTGA TCCGGGGCAGAATCCTGCACAACGGCGCCT ATTCTCTGACCCTGCAAGGCCTGGGAATCA GCTGGCTGGGACTGAGATCCCTGAGAGAGC	tHER2/ErbB 2		
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	TTGGATCTGGCCTGGCTCTGATCCACCACA ATACCCACCTGTGCTTCGTGCACACCGTGC CTTGGGACCAGCTGTTTCGGAATCCTCATC AGGCCCTGCTGCACACCGCCAACAGACCTG AGGATGAGTGTGTTGGCGAAGGCCTGGCTT GTCACCAGCTCTGTGCTAGAGGACACTGTT GGGGCCCTGGACCTACACAGTGCCTGAACT GTAGCCAGTTCCTGCGGGGCCAAGAGTGCG TGGAAGAGTGTAGAGTTCTGCAGGGACTGC CCCGGGAATACGTGAACGCCAGACACTGTC TGCCTTGTCACCCTGAGTGCCAGCCTCAGA ATGGCAGCGTGACCTGTTTTGGCCCTGAGG CCGATCAGTGCCTGGCCTGTGCTCACTACA AGGACCCTCCATTCTGCGTGGCCAGATGTC CTAGCGGCGTGAAGCCTGATCTGAGCTACA TGCCCATCTGGAAGTTCCCCGATGAGGAAG GCGCTTGCCAGCCTTGTCTATCAACTGCAC CCACAGCTGCGTGGACCTGGACGATAAGGG ATGTCCAGCCGAGCAGAGAGCCTCTCCACT GACCTCTATCATCTCTGCCGTCGTGGGCATC CTGCTGGTGGTGGTTCTGGGCGTTGTGTTCG GCATCCTGATTTGA			
95	DSPDRPWNPTFSPALLVVTEGDNATFTCSFS NTSESFVLNWYRMSPSNQDDRLAAFPEDRSQ PGQDARFRVTQLPNGRDFHMSVVRARRNDS GTYFCGAISLAPKAKIKESLRAELRVTERRAE VPTAHPSPSPRPAGQFQTPPCPSCPAPEFLGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEE MTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSGFFLYSRLTVDKSR WQEGNVFSCSVMHREALHNHYTQKSLSLSL GKASRKRRSGSGEGRGSLLTCGDVEENPGPM <i>LLLVTSLLLCELPHPAFLIPTQVCTGTDMKLRL</i> <i>PASPETHLDMLRHLYQGCQVVOGNLELTYPN</i> <i>ASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIV</i> <i>RGTQLFEDNYALAVLDNGDPLNNTTPVTGASPG</i>	PD1_4XMU T_M70_IgG 4Fc/T2A/tH ER2 IgG4Fc is bolded; furin cleavage site is double- underlined; T2A is underlined; and tEGFR is italic		

	GLRELQLRSLTEILKGGVLIQ^{RNP}Q^{LCYQ}DTILW KDIFHKNNQLALTLIDTNRSRACHPCSPMCKGS RCWGESSEDCQSLTRTV^{CAGGCARCKG}PLPTD CCHEQCAAGCTGPKHSDCLAC^{LHFNHSGICEL} HCPALVTYN^{DTFESMPNPEG}RYTFGASCVTAC PYN^YY^LSTDVGSCTLV^{CPLHNQE}VTAEDGTQ^{RCE} KCSKPCARVCYGLGMEHLREVRAVTSANIQEFA GCKKIFGSLAFLPESFDGDPASNTAPLQPEQLO VFETLEEITGYLYISAWPDSL^{PDLSVFQNLQ}VIRG RILHNGAYS^{LTLOGLGISWLGL}RSLRELGSGLALI HHNTHLCFVHTVPWDQ^{LF}RNP^{HQ}ALLHTANRP EDECVGEGLACHQ^{LCARGHCWGP}GPTQ^{CVNC} SQFLRGQECVEECRV^{LQGLPREYVNARHCLPCH} PECQPQNGSVTCFGPEADQCVACA^{HYKDPPFC} VARCPSGVKPDLSYMPIWKFPDEEGACQPCPIN CTHSCVDLDDKGC^{PAEQRASPLTSI}SAVVGILLV VVLGVVFGILI			
96	DSPDRPWN^{PPTF}SPALLV^{TEGD}NATFTCSFS NTSESV^{FLNWYRMSPSNQV}DRLAAFPEDRSQ PGQDARFRVTQLPNGRDFHMSVVRARRNDS GTYFCGAISLAPKWKIKESLRAELRV^{TERRPP} CPSCPAPEFLGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSQEDPEVQFNWYVDGVEVH NAKTKPREEQFNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKGLPSSIEKTISKAKGQPR EPQVYTLPPSQEEMTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTTTPVLDSGFS FLYSRLTVDKSRWQEGNVFSCSVMHEALH <u>NHYTQKSLSLSLGKASRKRRSGSGEGRGSLL</u> <u>TCGDVEENPGPMLLLVTSLLLCELPHPAFLLIPT</u> QVCTGTDMKLR^LPASPETHLDM^{LRHLYQGCQV} VQGNLELT^{YLP}TNASLSFLQ^{DIQEVOGYVLI}AHN QVRQVPLQRLRIVRG^{TQLFEDNYALAVLD}NGDP LNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQ RNPQ^{LCYQ}DTILWKDIFHKNNQLALTLIDTNRSR ACHPCSPMCKGSR^{CWGESSEDCQSLTRTV}CAG GCARCKG^{PLPTDCCHEQCAAGCTGPKHSDCLA} CLHFNHSGICELHCPALVTYN^{DTFESMPNPEG} RYTFGASCVTAC^{PYN}Y^LSTDVGSCTLV^{CPLHNQE} VTAEDGTQ^{RCE}KCSKPCARVCYGLGMEHLREV RAVTSANIQEFAGCKKIFGSLAFLPESFDGDPAS NTAPLQPEQLOVFETLEEITGYLYISAWPDSL^{PD} LSVFQNLQVIRGRILHNGAYS^{LTLOGLGISWLGL} RSLRELGSGLALIIHHNTHLCFVHTVPWDQ^{LF}RN PHQALLHTANRPED^{ECVGEGLACHQ}LCARGHC WGPGPTQ^{CVNC}SQFLRGQECVEECRV^{LQGLPR}	PD1_6XDM _IgG4Fc/T2 A/tHER2 IgG4Fc is bolded; furin cleavage site is double- underlined; T2A is underlined; and tEGFR is italic		

	<i>EYVNARHCLPCHPECQPONGSVTCFGPEADQC VACAHYKDPPFCVARCPSGVKPDLSYMPIWKFP DEEGACQPCPINCTHSCVDLDDKGCPAEQRAS PLTSIISAVVGILLVVVLGVVFGILI</i>			
97	MTTPRNSVNGTFPAEPMKGPIAMQSGPKPLF RRMSSLVGPTQSFFMRESKTLGAVQIMNGLF HIALGGLLMIPAGIYAPICVTWYPLWGGIMY IISGSLLAATEKNSRKCLVKGKMIMNSLSLFA AISGMILSIMDILNIKISHFLKMESLNFIRAHTP YINIYNCEPANPSEKNPSTQYCYSIQSLFLGIL SVMLIFAFFQELVIAGIVENEWKRTCSRPKSNI VLLSAEEKKEQTIEIKEEVVGLTETSSQPKNEE DIEIPIQEEEEETETNFPEPPQDQESSPIENDS SP	CD20 (297 amino acids)		
98	ATGACAACACCTAGAAATAGCGTGAACGGC ACATTCCCCGCCGAGCCTATGAAGGGACCT ATCGCCATGCAGAGCGGCCCAAGCCTCTG TTAGACGGATGTCTAGCCTCGTGGGCCCC ACACAGAGCTTTTTCATGAGAGAGAGCAAG ACCCTGGGCGCCGTGCAGATCATGAACGGC CTGTTTCACATTGCCCTCGGCGGCCTGCTGA TGATCCCTGCCGGAATCTATGCCCTATCTG CGTGACCGTGTGGTATCCTCTGTGGGGCGG CATCATGTACATCATCTCTGGATCTCTGCTG GCCGCCACCGAGAAGAACAGCAGAAAGTG TCTGGTCAAGGGCAAGATGATCATGAATAG CCTGAGCCTGTTCGCCGCCATCAGCGGCAT GATCCTGAGCATCATGGATATCCTGAATAT CAAGATCAGCCACTTCCTGAAGATGGAAAG CCTGAACTTCATCAGGGCCCACACACCTTA CATCAACATCTACAACCTGCGAGCCCGCCAA TCCTAGCGAGAAGAATAGCCCCAGCACACA GTACTGCTACTCTATCCAGAGCCTGTTTCTG GGCATCCTGAGCGTGATGCTGATCTTCGCA TTCTTCCAAGAGCTGGTTATCGCCGGCATC GTGGAAAACGAGTGGAAGCGGACCTGCAG CAGACCCAAGAGCAACATCGTGCTGCTGAG CGCCGAGGAAAAGAAAGAGCAGACCATCG AGATCAAAGAGGAAGTCGTCGGCCTGACCG AGACAAGCAGCCAGCCTAAGAACGAAGAG GACATTGAGATCATCCCCATCCAAGAAGAG GAAGAAGAAGAGACTGAGACAACTTCCC CGAGCCTCCTCAGGACCAAGAGAGCAGCCC CATTGAGAACGACAGCAGCCCTTGA	CD20		

99	DSPDRPWNPTFSPALLVVTEGDNATFTCSFS NTSESFVLNWYRMSPSNQDDRLAAFPEDRSQ PGQDARFRVTQLPNGRDFHMSVVRARRNDS GTYFCGAISLAPKAKIKESLRAELRVERRAE VPTAHPSPSRPAGQFQTPPCPSCPAPEFLGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEE MTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSRLTVDKSR WQEGNVFSCSVMHEALHNHYTQKSLSL GKASRKRRSGSGEGRGSLLTCGDVEENPGPM <i>TTPRNSVNGTFPAEPMKGPIAMQSGPKPLFRM</i> <i>SSLVGPTQSFFMRESKTLGAVQIMNGLFHIALG</i> <i>GLLMIPAGIYAPICVTVWYPLWGGIMYIISGSLA</i> <i>ATEKNSRKCLVKGKMIMNSLSLFAAISGMILSIM</i> <i>DILNIKISHFLKMESLNFIRAHTPYINIYNCEPANP</i> <i>SEKNSPSTQYCYSIQSLFLGILSVMLIAFFQELVI</i> <i>AGIVENEWKRTCSPKSNIVLLSAEEKKEQTIEIK</i> <i>EEVVGLTETSSQPKNEEDIEIPIQEEEEETETN</i> <i>FPEPPQDQESSPIENDSSP</i>	PD1_4XMU T_M70_IgG 4Fc/T2A/CD 20 IgG4Fc is bolded; furin cleavage site is double- underlined; T2A is underlined; and tEGFR is italic		
100	DSPDRPWNPTFSPALLVVTEGDNATFTCSFS NTSESFVLNWYRMSPSNQDDRLAAFPEDRSQ PGQDARFRVTQLPNGRDFHMSVVRARRNDS GTYFCGAISLAPKAKIKESLRAELRVERRAE VPTAHPSPSRPAGQFQTPPCPSCPAPEFLGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFN STYRVVSVLTVLHQDWLNGKEYKCKVSNK GLPSSIEKTISKAKGQPREPQVYTLPPSQEE MTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSRLTVDKSR WQEGNVFSCSVMHEALHNHYTQKSLSL GKASRKRRSGSGEGRGSLLTCGDVEENPGPM <i>TTPRNSVNGTFPAEPMKGPIAMQSGPKPLFRM</i> <i>SSLVGPTQSFFMRESKTLGAVQIMNGLFHIALG</i> <i>GLLMIPAGIYAPICVTVWYPLWGGIMYIISGSLA</i> <i>ATEKNSRKCLVKGKMIMNSLSLFAAISGMILSIM</i> <i>DILNIKISHFLKMESLNFIRAHTPYINIYNCEPANP</i> <i>SEKNSPSTQYCYSIQSLFLGILSVMLIAFFQELVI</i> <i>AGIVENEWKRTCSPKSNIVLLSAEEKKEQTIEIK</i> <i>EEVVGLTETSSQPKNEEDIEIPIQEEEEETETN</i> <i>FPEPPQDQESSPIENDSSP</i>	PD1_6XDM _IgG4Fc/T2 A/CD20 IgG4Fc is bolded; furin cleavage site is double- underlined; T2A is underlined; and tEGFR is italic		

CLAIMS

What is claimed is:

1. A programmed cell death 1 (PD-1) decoy polypeptide, comprising an amino acid
5 sequence of a PD-1 variant, wherein the amino acid sequence has at least 80% identity to SEQ
ID NO: 6 and comprises substitutions at least at positions T76, K78, and Q133 of SEQ ID NO: 6.

2. The PD-1 decoy polypeptide of claim 1, wherein the substitutions comprise additional
substitutions at least at one of positions L122 and A132.

10 3. The PD-1 decoy polypeptide of any one of the preceding claims, wherein the
substitutions comprise:

(i) T76D or a conservative substitution of D76; or T76V or a conservative
substitution V76;

15 (ii) K78R or a conservative substitution of R78; or

(iii) Q133K or a conservative substitution of K133.

4. The PD-1 decoy polypeptide of any one of the preceding claims, wherein the
additional substitutions comprise at least one of:

20 (iv) L122E or a conservative substitution of E122; L122F or a conservative
substitution of F122; or L122I or a conservative substitution of I122; and

(v) A132W or a conservative substitution of W132; A132L or a conservative
substitution of L132; or A132Y or a conservative substitution of Y132.

25 5. The PD-1 decoy polypeptide of any one of the preceding claims, wherein the amino
acid sequence comprises the substitutions at the positions at T76, K78, E122, A132, and Q133.

6. The PD-1 decoy polypeptide of any one of the preceding claims, wherein the amino
acid sequence comprises the substitutions at the positions at T76, K78, E122, and Q133.

7. The PD-1 decoy polypeptide of any one of claims 1 to 5, wherein the substitutions comprise:

- (a) T76D, K78R, L122F, and Q133K; or
- (b) T76V, K78R, L122F, A132W, and Q133K.

5

8. The PD-1 decoy polypeptide of any one of the preceding claims, further comprising a fusion partner.

9. The PD-1 decoy polypeptide of claim 8, wherein the fusion partner comprises a
10 fragment of a human immunoglobulin polypeptide sequence.

10. The PD-1 decoy polypeptide of claim 9, wherein the fragment comprises: (a) a CH3 domain; and (b) part or whole of an Fc region.

11. The PD-1 decoy polypeptide of claim 9, wherein the fragment comprises a human
15 IgG Fc sequence.

12. The PD-1 decoy polypeptide of any one of the preceding claims, further comprising a cellular elimination tag (CET).

20

13. The PD-1 decoy polypeptide of claim 12, wherein the CET comprises a truncated EGFR (tEGFR) or a variant thereof, a truncated HER2 (tHER2) or a variant thereof, a CD20 or a variant thereof, or a CD19 or a variant thereof.

14. The PD-1 decoy polypeptide of claim 13, wherein the fusion partner is linked to the
25 C-terminus of the amino acid sequence of the PD-1 variant and the CET is linked to the C-terminus of the fusion partner.

15. The PD-1 decoy polypeptide of claim 1, comprising an amino acid sequence selected
30 from SEQ ID NOs: 9-65, 90, 92, 95-96, and 99-100.

16. The PD-1 decoy polypeptide of claim 13, wherein: the tEGFR comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 88 or comprises the amino acid sequence of SEQ ID NO: 88; the HER2 comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 93 or comprises the amino acid sequence of SEQ ID NO: 93; and the
5 CD20 comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 98 or comprises the amino acid sequence of SEQ ID NO: 98.

17. The PD-1 decoy polypeptide of any one of the preceding claims, comprising a detectable label.

10 18. A polynucleotide comprising a polynucleotide sequence that encodes the PD-1 decoy polypeptide of any one of the preceding claims.

19. The polynucleotide of claim 18, further comprising: (a) a nucleotide sequence
15 encoding an alpha polypeptide and a beta polypeptide of a T cell receptor (TCR); or (b) a nucleotide sequence encoding a chimeric antigen receptor (CAR).

20. A vector comprising the polynucleotide of any one of claims 18 to 19.

20 21. A cell comprising the polynucleotide of claim 18 or 19 or the vector of claim 20.

22. The cell of any one of claims 21 to 23, wherein the cell further comprises a second polynucleotide sequence encoding at least one transgene.

25 23. The cell of claim 22, wherein the transgene is selected from an IL-2 variant, LIGHT or a variant thereof, IL-33 or a variant thereof, and CD40L or a variant thereof.

24. The cell of claim 22, wherein the polynucleotide and the second polynucleotide are on the same vector.

30 25. The cell of any one of claims 21 to 24, wherein the cell is a lymphocyte.

26. The cell of claim 25, wherein the lymphocyte expresses:

- (a) the PD-1 decoy or the variant thereof and tEGFR or the variant thereof;
- (b) the PD-1 decoy or the variant thereof and the IL-2 variant;
- 5 (c) the PD-1 decoy or the variant thereof and the LIGHT or the variant thereof;
- (d) the PD-1 decoy or the variant thereof and the IL-33 or the variant thereof;
- (e) the PD-1 decoy or the variant thereof and the CD40L or the variant thereof;
- (f) the PD-1 decoy or the variant thereof, the IL-2 variant, and the IL-33 or the variant thereof;
- 10 (g) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the IL-2 variant;
- (h) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the LIGHT or the variant thereof;
- (i) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the IL-15 33 or the variant thereof;
- (j) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, and the CD40L or the variant thereof;
- (k) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-2 variant, and the IL-33 or the variant thereof;
- 20 (l) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-2 variant, and the CD40L or the variant thereof; or
- (m) the PD-1 decoy or the variant thereof, the tEGFR or the variant thereof, the IL-33 variant, and the CD40L or the variant thereof.

25 27. The cell of any one of claims 25 to 26, wherein the lymphocyte is autologous.

28. The cell of any one of claims 25 to 27, wherein the lymphocyte is a tumor-infiltrating lymphocyte (TIL).

30 29. The cell of any one of claims 25 to 28, wherein the lymphocyte expresses a chimeric antigen receptor (CAR) or a recombinant T cell receptor (TCR).

30. The cell of claim 29, wherein the recombinant T cell receptor (TCR) shows reactivity against NY-ESO1, MAGE-A1, MAGE-A3, MAGE A-10, MAGE-C2, SSX2, MAGE-A12, or a combination thereof.

5

31. A composition comprising: (i) the polypeptide of any one of claims 1 to 17; (ii) the polynucleotide of any one of claims 18 to 19; (iii) the vector of claim 20; or (iv) the cell of claims 21 to 30.

10

32. A pharmaceutical composition comprising an effective amount of the composition of claim 31 and a pharmaceutically acceptable carrier.

33. The pharmaceutical composition of claim 32, further comprising a second therapeutic agent.

15

34. A kit comprising an effective amount of the composition of claim 31 or the pharmaceutical composition of claim 32 or 33.

35. A method of treating a cancer or tumor in a subject, comprising administering to a subject in need thereof a therapeutically effective amount of the composition of claim 31 or the pharmaceutical composition of claim 32 or 33.

20

36. The method of claim 35, the cancer is selected from melanoma, sarcoma, ovarian cancer, prostate cancer, lung cancer, bladder cancer, MSI-high tumors, head and neck tumors, kidney cancer, and breast cancer.

25

37. The method of claim 35 or 36, further comprising administering to the subject a second therapeutic agent or therapy.

30

38. The method of claim 37, wherein the second therapeutic agent comprises an anti-cancer or anti-tumor agent.

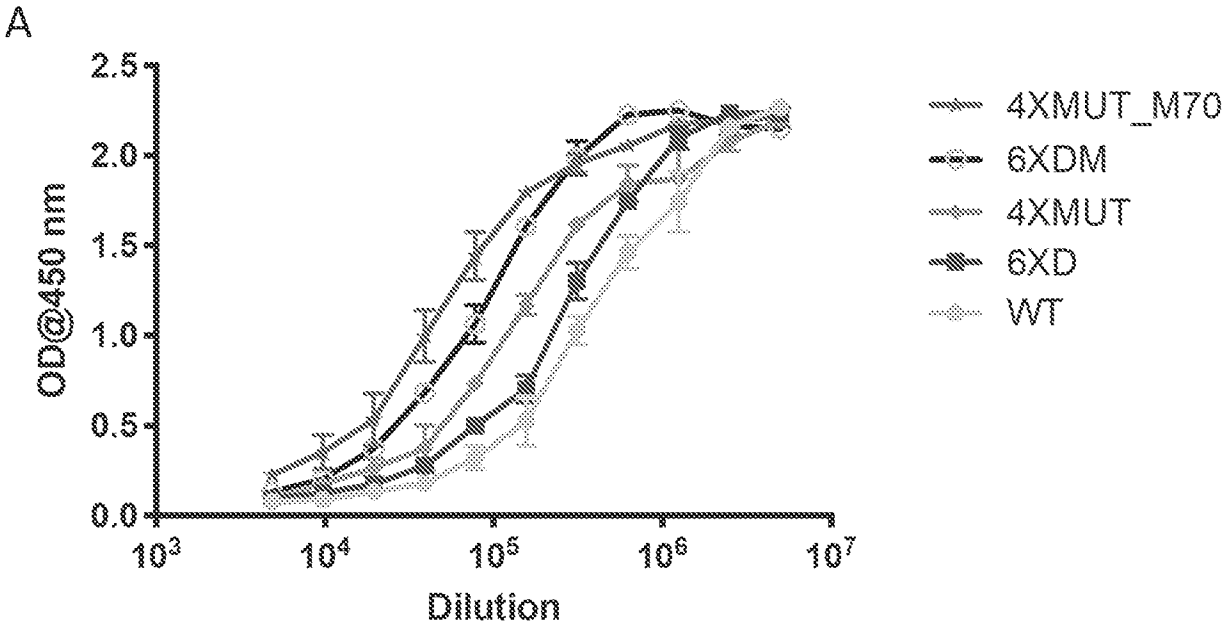
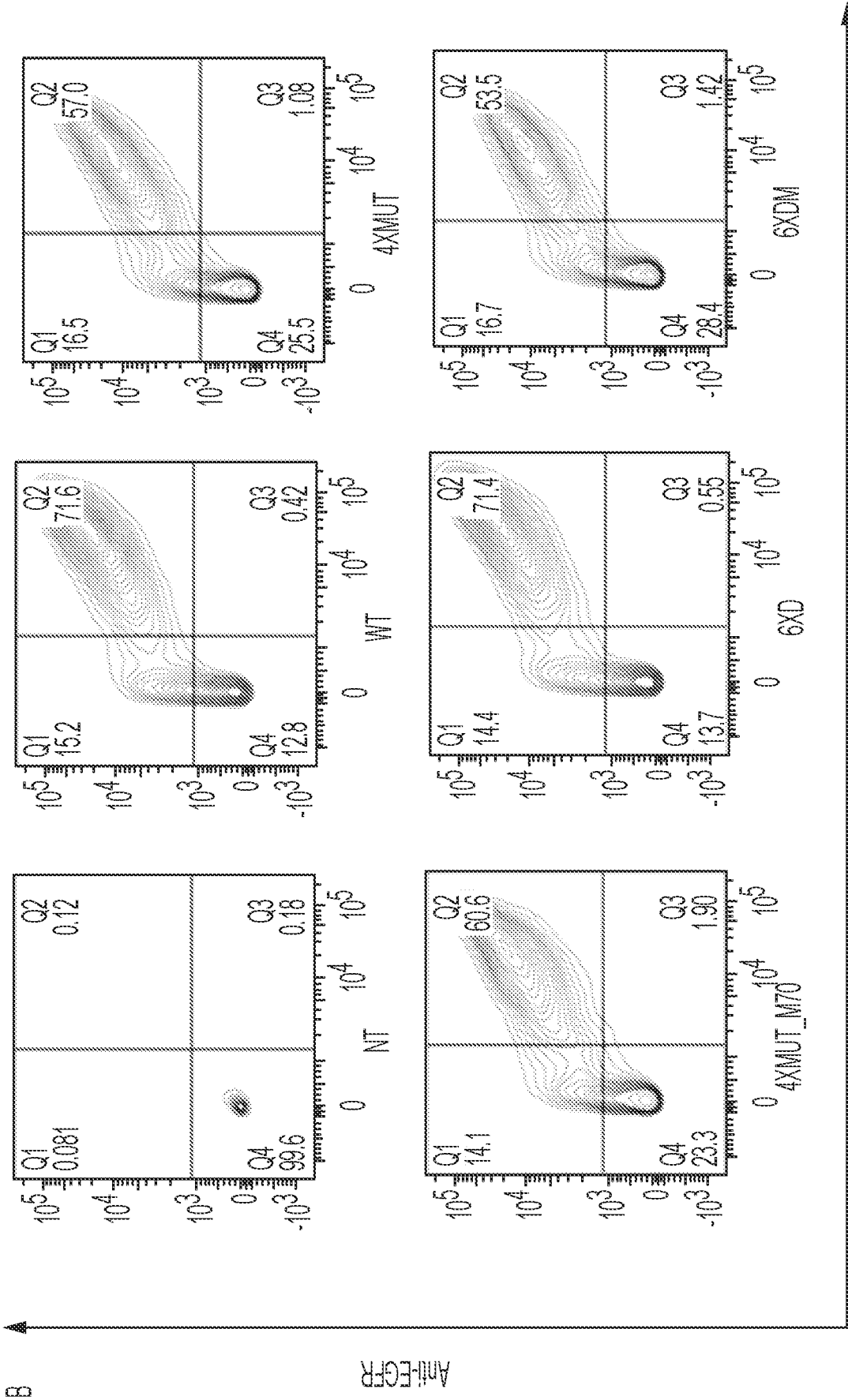


FIG. 1A



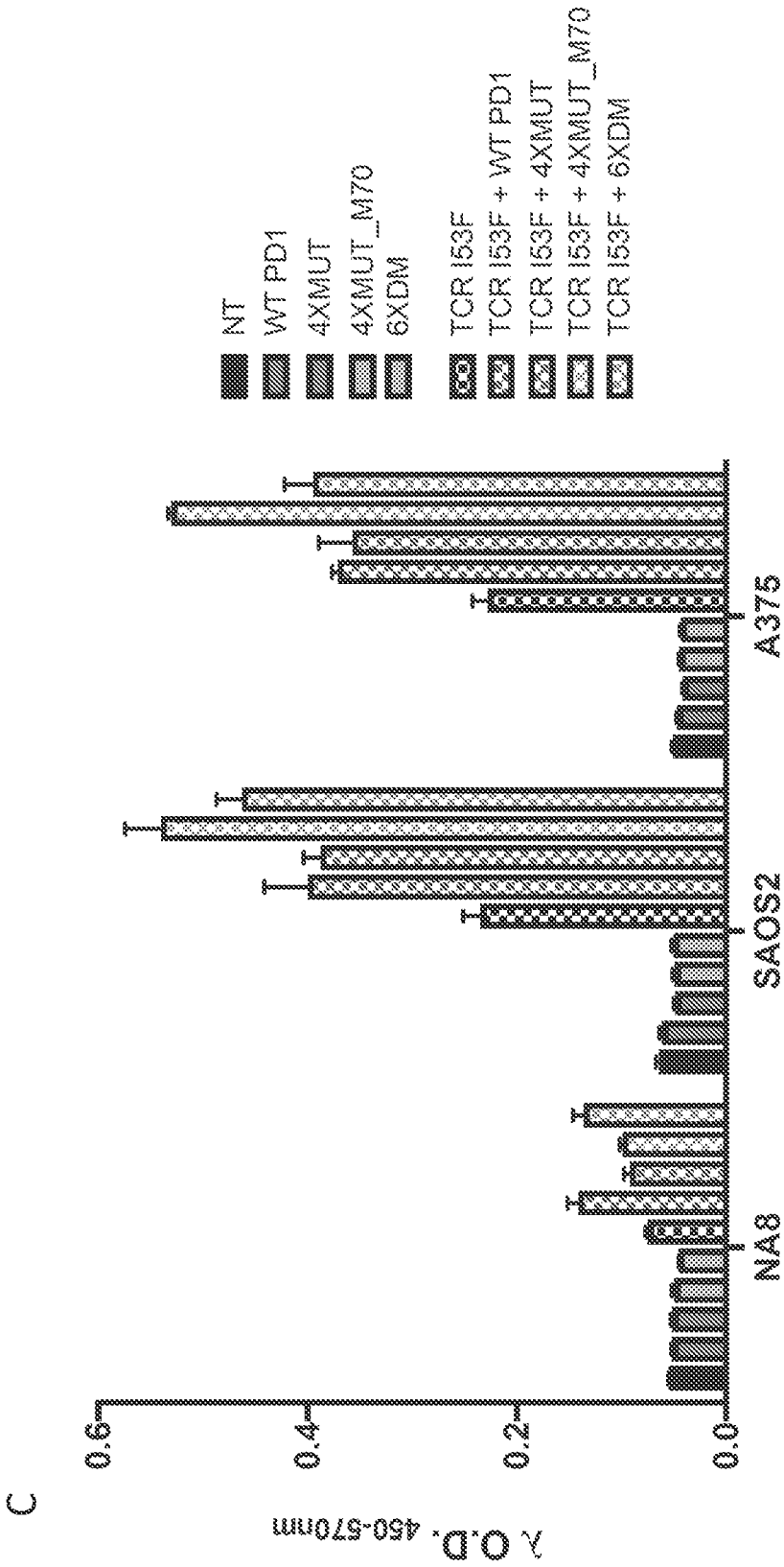


FIG. 1C

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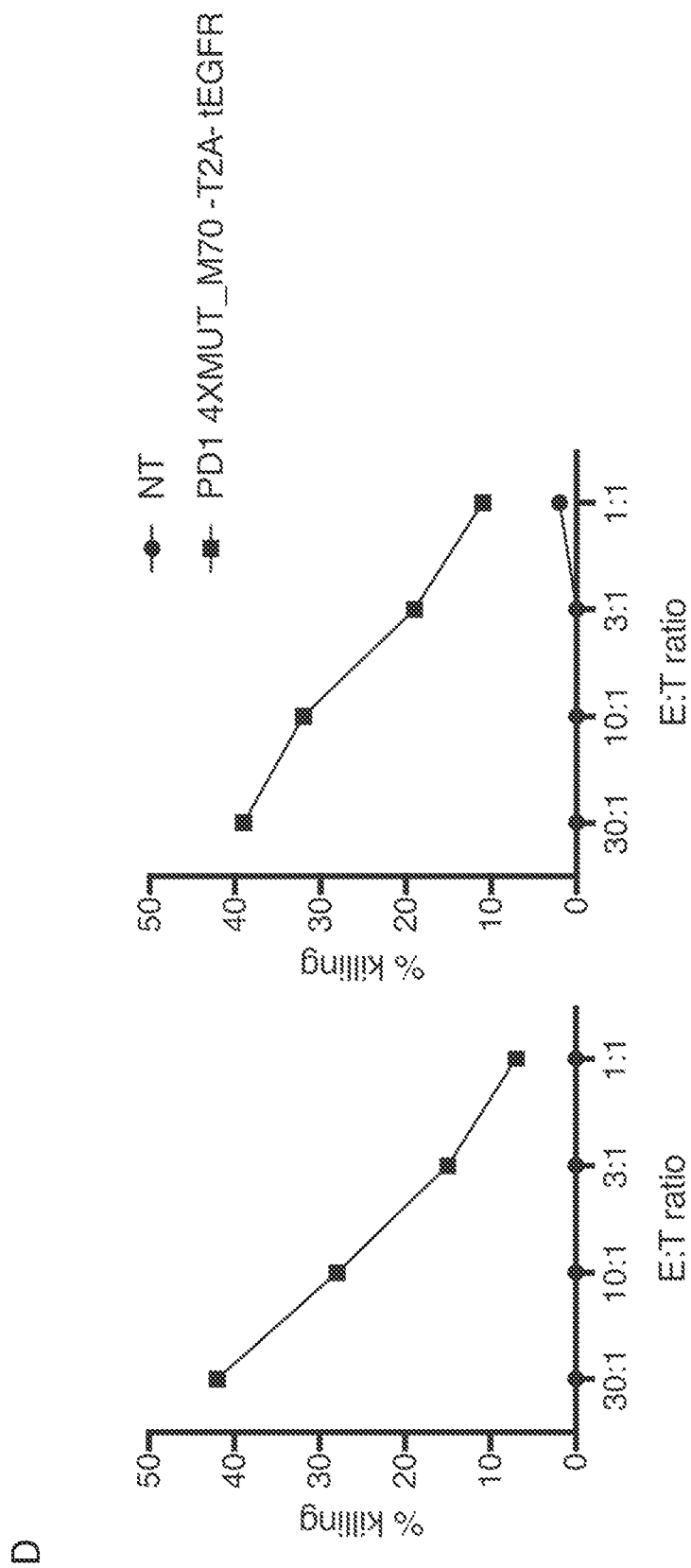
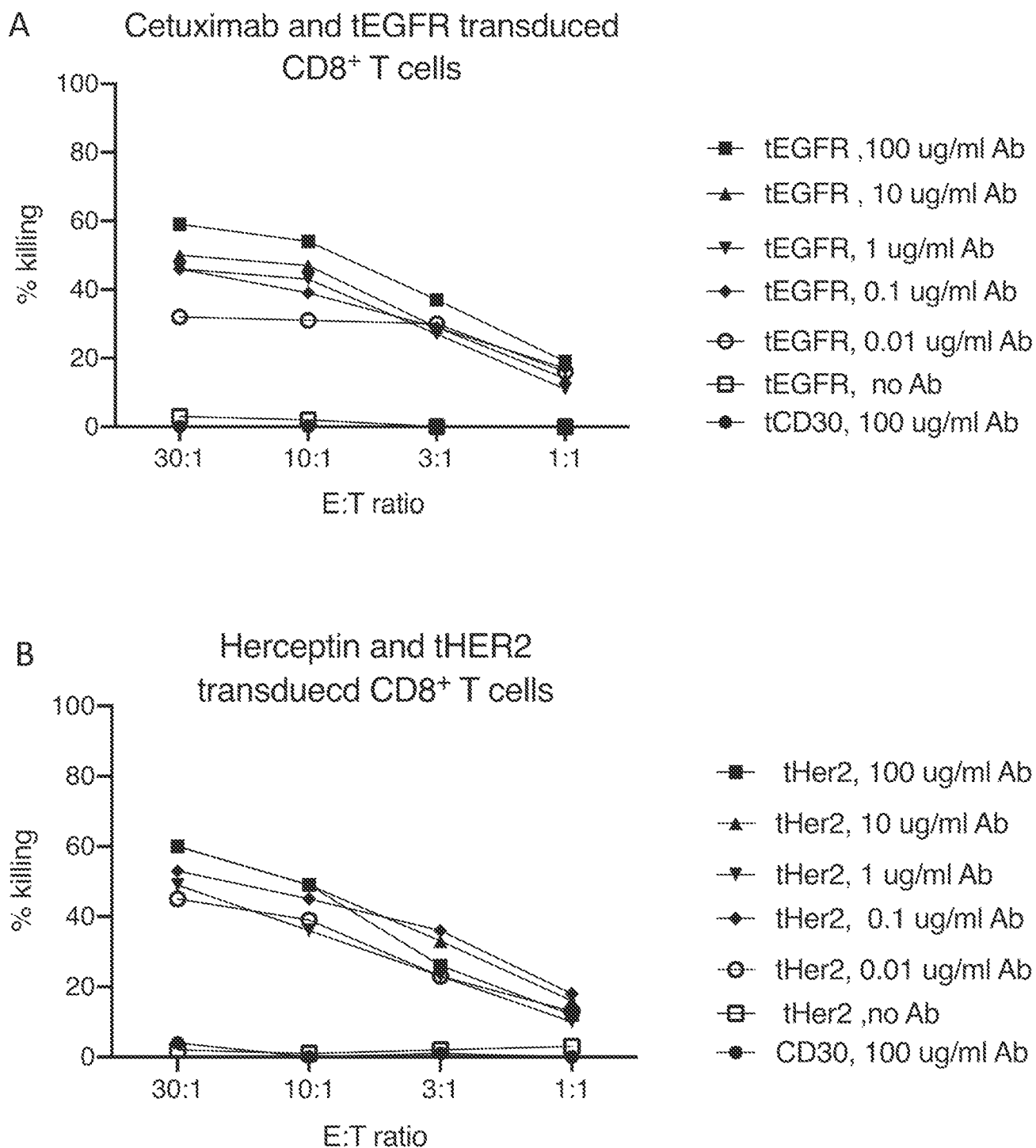


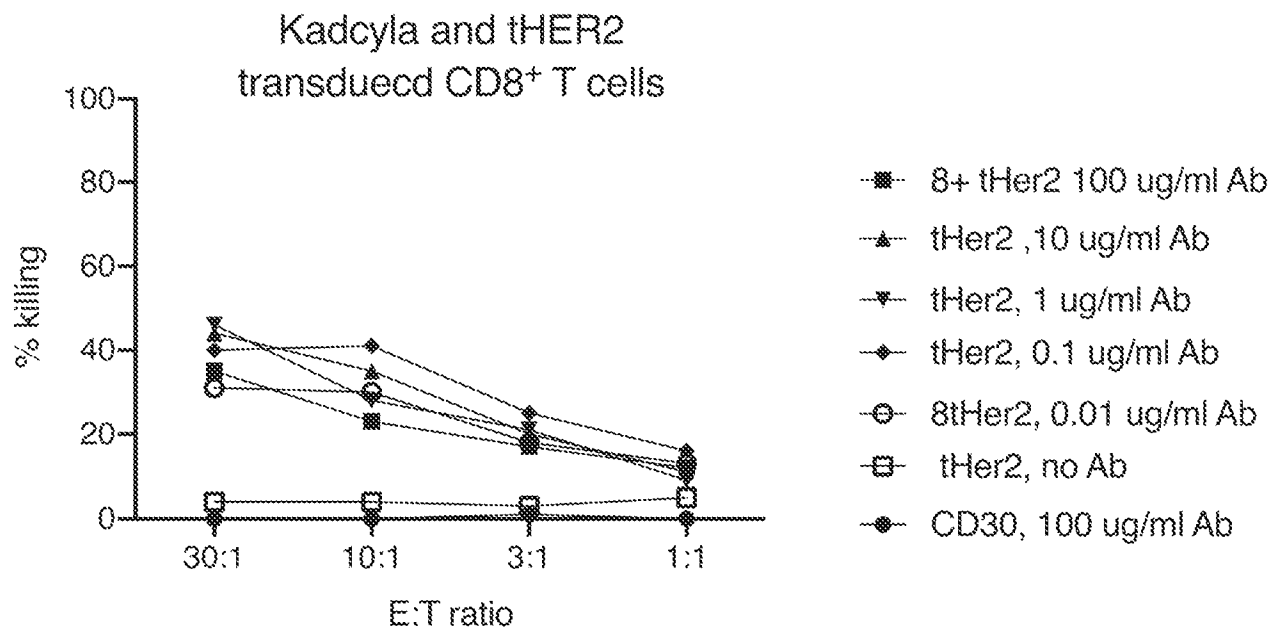
FIG. 1D

5/10

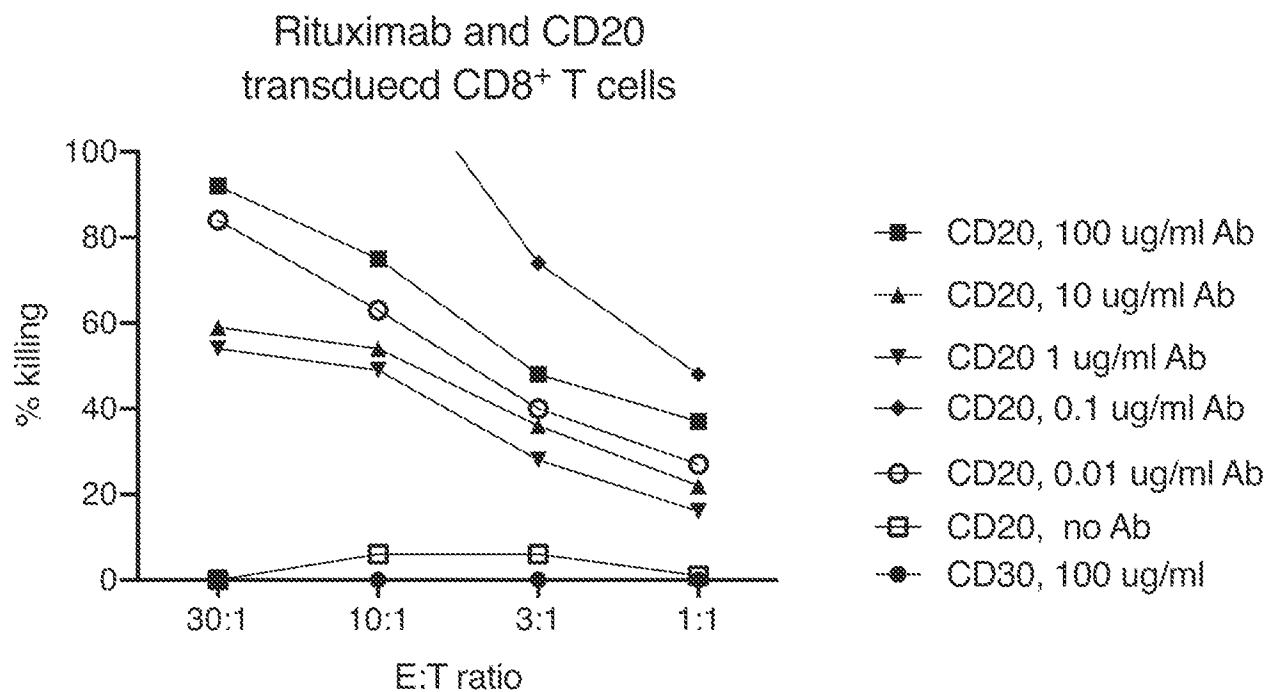
**FIGS. 2A and 2B**

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C



D



FIGS. 2C and 2D

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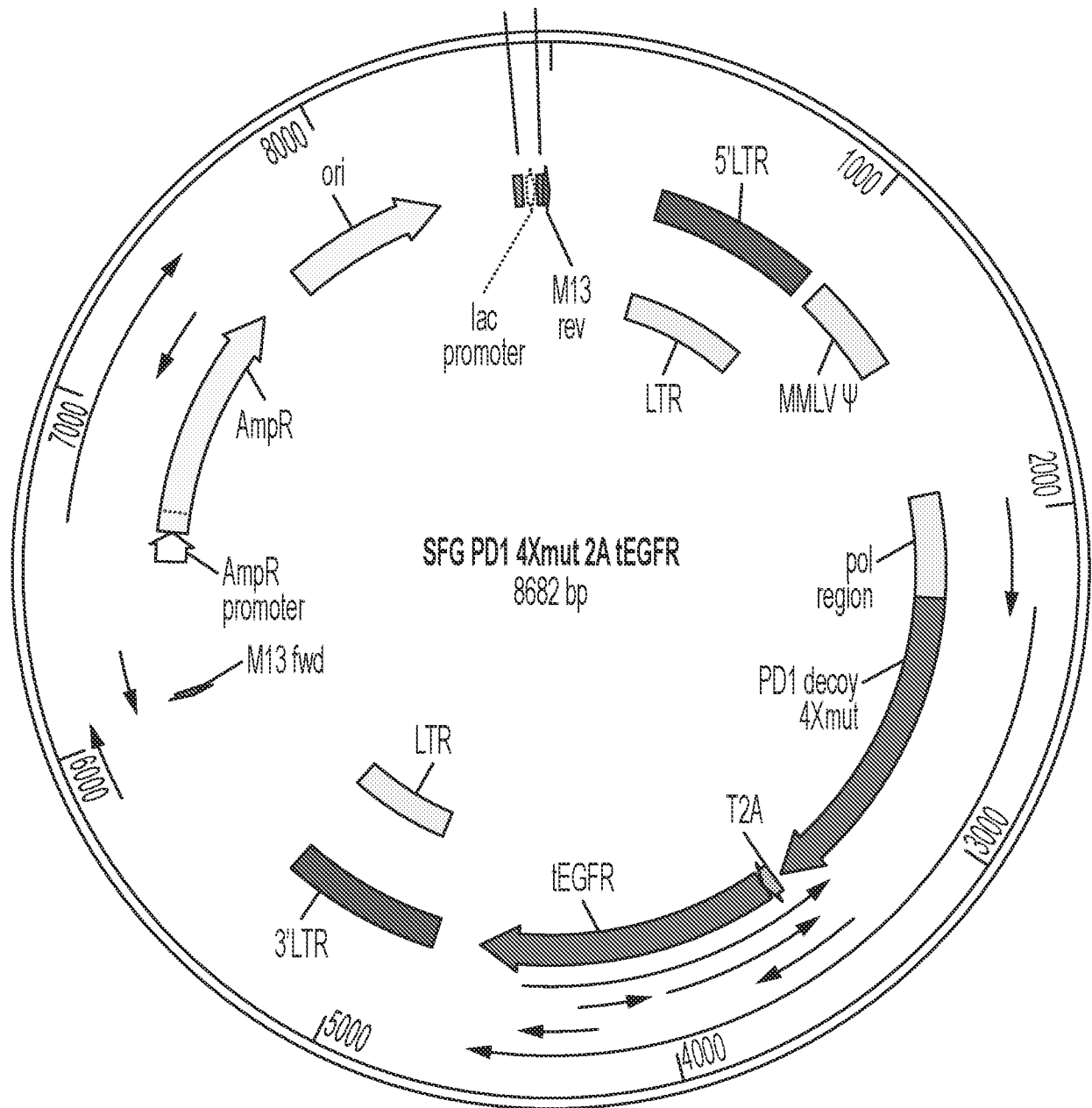


FIG. 3A

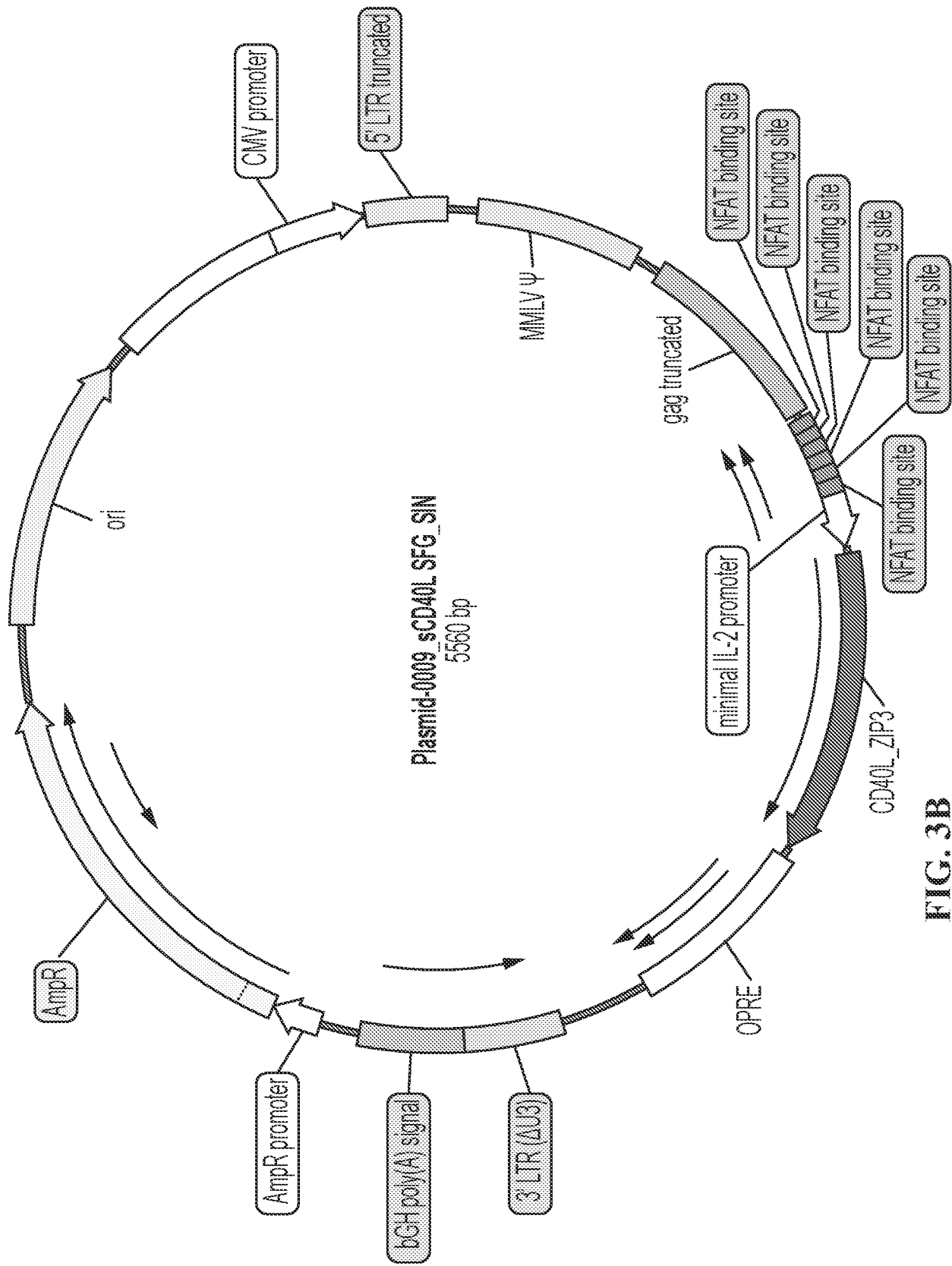


FIG. 3B

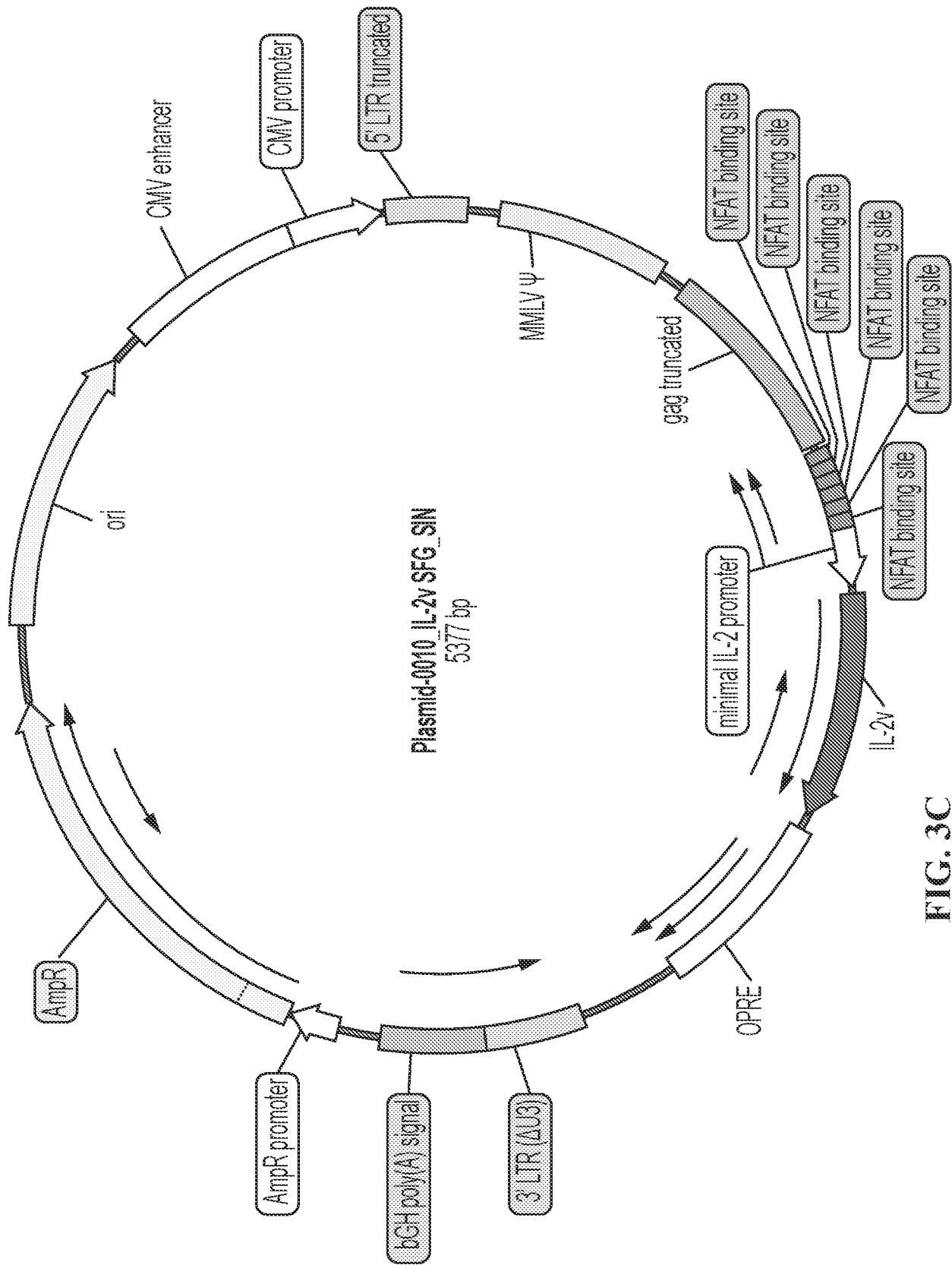


FIG. 3C

10/10

MASDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSES
23| 30| 40| 50| 60|
FVLNWYRMSPSNQTDKLAAPEDRSQPGQDARFRVTQLPN
70| 80| 90| 100|
GRDFHMSVVRARRNDSTYLCGAISLAPKAQIKESLRAEL
110| 120| 130| 140|
RVTERRALE
150| 151|

(SEQ ID NO: 6)

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/59119

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/16, A61K 38/00, A61P 35/00, C07K 14/00 (2022.01)

CPC - A61K 38/16, A61K 38/00, A61P 35/00, C07K 14/00, C07K 2319/03

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.
Y	WO 2019/241758 A1 (ALPINE IMMUNE SCIENCES, INC.) 19 December 2019 (19.12.2019) para [0061]; claim 1; claim 23	1-3
A		15
Y	US 2019/0381184 A1 (THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL) 19 December 2019 (19.12.2019) claim 128; SEQ ID NO: 28	1-3
A		15
Y	US 2016/0052990 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR) 25 February 2016 (25.02.2016) Abstract; para [0021]	2, 3/2
Y	SHRESTHA et al. "Computational Redesign of PD-1 Interface for PD-L1 Ligand Selectivity" Structure, 7 May 2019, Vol. 27, No. 5, pp 829-836 especially, page 832, Col. 1, para 2; page 832, Col. 2, para 1	3
A	US 2018/0185668 A1 (REGENERON PHARMACEUTICALS INC.) 5 July 2018 (05.07.2018) para [0061]; SEQ ID NO: 321	15

☐ 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

28 February 2022

Date of mailing of the international search report

MAR 25 2022

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Kari Rodriguez

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/59119

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 13ter.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 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.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:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/59119

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

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

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

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

3. ☒ Claims Nos.: 4-14, 16-38
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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

-----continued on supplemental sheet-----

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.:
1-3, 15, limited to additional substitution at position L122, the substitutions comprise T76D and wherein PD-1 decoy polypeptide with sequence of SEQ ID NO: 9 & SEQ ID NO: 54
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.

Box III: Observations where unity of invention is lacking:

Group I+: 1-3 and 15 directed to a programmed cell death 1 (PD-1) decoy polypeptide. The PD-1 decoy polypeptide will be searched to the extent wherein the additional substitution comprise substitution at position L122, wherein the substitutions comprise: (i) T76D and wherein PD-1 decoy polypeptide comprises an amino acid sequence of SEQ ID NO: 9. It is believed that claims 1-3 and 15 encompass this first named invention, and thus these claims will be searched without fee to the extent that PD-1 decoy polypeptide comprises additional substitution comprise substitution at position L122, wherein the substitutions comprise: (i) T76D and wherein PD-1 decoy polypeptide comprises an amino acid sequence of SEQ ID NO: 9. Additional PD-1 decoy polypeptide will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected PD-1 decoy polypeptide. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a PD-1 decoy polypeptide comprising additional substitution comprise substitution at position A132, wherein the substitutions comprise: (ii) K78R and wherein PD-1 decoy polypeptide comprises an amino acid sequence of SEQ ID NO: 10 (claims 1-3, 15).

The inventions listed as Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I+ requires a PD-1 decoy polypeptide, wherein each invention requires a sequence of PD-1 decoy polypeptide, not required by any of the other inventions.

Common Technical Features

The features shared by the inventions listed as Group I+ are a programmed cell death 1 (PD-1) decoy polypeptide, comprising an amino acid sequence of a PD-1 variant, and comprises substitutions at least at positions T76, K78, and Q133.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by WO 2019/241758 A1 to Alpine Immune Sciences, Inc. (hereinafter 'Alpine').

Alpine discloses (instant claim 1) a programmed cell death 1 (PD-1) decoy polypeptide, comprising an amino acid sequence of a PD-1 variant, and comprises substitutions at least at positions 76, 78, and 133 (para [0061]- "the cognate binding partner of the transmembrane bound vlgD is an inhibitory ligand (e.g. PD-L1), and the TIP containing the vlgD (e.g. PD-1 vlgD) can act as a decoy counter- structure to inhibit specific binding by and between PD-I and PD-L1. The PD-1 TIP?"; claim 1; claim 10- "wherein at least one amino acid modification is T133A, T133R or T133S"; claim 23- "amino acid modifications selected from...R76H, R76S, V77D, T78S"), but does not teach comprises substitutions at least at positions T76, K78, and Q133. However, Alpine teaches substitutions at positions 76, 78, and 133 (claim 1; claim 10- "wherein at least one amino acid modification is T133A, T133R or T133S"; claim 23- "amino acid modifications selected from...R76H, R76S, V77D, T78S"). Therefore, it would have been obvious to one of ordinary skill in the art, to have used the teachings of Alpine and optimized the substitutions at 76, K78, and Q133, to achieve a desired polypeptide property, using routine experimentation.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Groups I+ therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Item 4 (continued)

Claims 4-14 and 16-38 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

NOTE:

The amino acid positions in the claims do not correspond to the amino acid positions in SEQ ID NO:6. See Figure 4 wherein amino acid 1 of SEQ ID NO:6 is identified as 23, as in the numbering starts with 23 instead of 1. For this reason, amino acid position "T76" of Figure 4 and the claims is construed as "T54" of SEQ ID NO:6; amino acid position "K78" of Figure 4 and the claims is construed as "K56" of SEQ ID NO:6; amino acid position "Q133" of Figure 4 and the claims is construed as "Q111" of SEQ ID NO:6; amino acid position "L122" of Figure 4 and the claims is construed as "L100" of SEQ ID NO:6; amino acid position "A132" of Figure 4 and the claims is construed as "A110" of SEQ ID NO:6.