(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 January 2017 (05.01.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/004150 A1

(51) **International Patent Classification**:
C07K 19/00 (2006.01) ***C07K 14/725*** (2006.01)
C12N 15/62 (2006.01) ***A61K 38/17*** (2006.01)
C07K 14/705 (2006.01) ***A61P 35/00*** (2006.01)

(21) **International Application Number**:
PCT/US2016/040010

(22) **International Filing Date**:
29 June 2016 (29.06.2016)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
62/186,108 29 June 2015 (29.06.2015) US

(71) **Applicant**: **THE JOHNS HOPKINS UNIVERSITY** [US/US]; 3400 North Charles Street, Baltimore, MD 21218 (US).

(72) **Inventors; and**
(71) **Applicants** *(for US only)*: **BORRELLO, Ivan, M.** [US/US]; 912 Kingston Road, Baltimore, MD 21231 (US). **LEE, Susan** [US/US]; 828 S Highland Avenue, Baltimore, MD 21224 (US). **NOONAN, Kimberly, A.** [US/US]; 6607 Bonnie Ridge Drive, Apt 102, Baltimore, MD 21209 (US). **PARDOLL, Drew, M.** [US/US]; 19400 James Creek Court, Brookeville, MD 20833-2241 (US).

(74) **Agents**: **RUSSELL, Hathaway, P.** et al.; Foley Hoag LLP, Seaport West, 155 Seaport Boulevard, Boston, MA 02210-2600 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

WO 2017/004150 A1

(54) **Title**: IMMUNE CHECKPOINT CHIMERIC ANTIGEN RECEPTORS THERAPY

(57) **Abstract**: In some aspects, the embodiments relate to compositions and methods related to chimeric transmembrane proteins. The chimeric transmembrane proteins may comprise the extracellular domain of an inhibitory receptor, and an intracellular signaling domain that can activate an immune response.

IMMUNE CHECKPOINT CHIMERIC RECEPTORS THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/186,108, filed June 29, 2015, which is hereby incorporated by reference in its entirety.

BACKGROUND

The large majority of patients with malignancies will die from their disease. One approach to treating these patients is to genetically modify T cells to target antigens expressed on tumor cells through the expression of chimeric antigen receptors (CARs). CARs are antigen receptors that are designed to recognize cell surface antigens in a human leukocyte antigen-independent manner. Outside of the successes with CD19-targeted approaches, attempts at using genetically modified cells expressing CARs to treat other malignancies have met with limited success.

Recently, checkpoint inhibiting antibodies targeting CTLA-4 (ipilimumab) and PD-1 (nivolumab, pembrolizumab) have shown considerable activity in the treatment of various malignancies including metastatic melanoma, non small cell lung cancer (NSCLC) and Hodgkin's lymphoma. These data demonstrate how checkpoint blockade represents a major obstacle to effective immunotherapy by overcoming T cell anergy.

SUMMARY

In some aspects, the embodiments relate to a chimeric transmembrane protein, comprising the extracellular domain of an inhibitory receptor and an intracellular signaling domain that can activate an immune response. The extracellular domain may be, for example, an extracellular domain from CTLA-4, PD-1, LAG-3, or Tim-3. The intracellular signaling domain may be, for example, the intracellular signaling domain of CD3ζ, 4-1BB, or CD28. In some aspects, the embodiments relate to a nucleic acid encoding a chimeric transmembrane protein as described herein.

In some aspects, the embodiments relate to cells, comprising a nucleic acid encoding a chimeric transmembrane protein as described herein. In some aspects, the embodiments relates to cells, comprising a chimeric transmembrane protein as described herein.

In some aspects, the embodiments relate to methods for making recombinant cells, comprising transfecting cells with a nucleic acid encoding a chimeric transmembrane protein as described herein.

In some aspects, the embodiments relate to methods for increasing an immune response in a subject, comprising administering to the subject a recombinant cell as described herein. In some aspects, the embodiments relate to methods for treating a neoplasm in a subject, comprising administering to the subject a recombinant cell as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a nucleotide sequence (SEQ ID NO:1) encoding a chimeric transmembrane protein, comprising a leader peptide from CD8 ("CD8a LP"), the extracellular domain of mouse PD-1 ("PD-1 ECD"), and the transmembrane and intracellular domains of mouse 4-1BB ("4-1BB TM" and "4-1BB ICD", respectively). The reverse complement of the nucleotide sequence (SEQ ID NO:2) is also shown. Codons were optimized for expression in mouse lymphocytes.

Figure 2 shows a nucleotide sequence (SEQ ID NO:3) encoding a chimeric transmembrane protein, comprising a leader peptide from CD8 ("CD8a LP"), the extracellular domain of human PD-1 ("PD-1 ECD"), and the transmembrane and intracellular domains of human 4-1BB ("4-1BB TM" and "4-1BB ICD", respectively). The reverse complement of the nucleotide sequence (SEQ ID NO:4) is also shown. Codons were optimized for expression in human lymphocytes.

Figure 3 shows flow cytometry results for Lenti-X 293T cells transfected with a mCherry gene and a nucleic acid encoding a chimeric transmembrane protein (SEQ ID NO:1), comprising the extracellular domain of PD-1 using the transfection protocol described in Example 2, *infra*. Figure 3 shows that the nucleic acid is expressed in 293T cells.

Figure 4 shows flow cytometry results for Lenti-X 293T cells transduced with a mCherry gene and a nucleic acid encoding a chimeric transmembrane protein (SEQ ID NO:1), comprising the extracellular domain of PD-1 and the intracellular domain of 4-1BB, using the transduction protocol described in Example 1, *infra*. Cells were transduced in 1 well of a 6-well plate with 1.9mL of virus. Figure 4 shows that the nucleic acid is expressed in 293T cells.

Figure 5 shows flow cytometry results for Lenti-X 293T cells transduced with a mCherry gene and a nucleic acid encoding a chimeric transmembrane protein (SEQ ID NO:1), comprising the extracellular domain of PD-1 and the intracellular domain of 4-1BB, using the transduction protocol described in Example 1, *infra*. Cells were transduced in 1

well of a 6-well plate with 0.38mL of virus. Figure 5 shows that the nucleic acid is expressed in 293T cells.

Figure 6, Panel A and Panel B illustrates that MILs comprising a chimeric receptor having a PD-1 extracellular domain, a 4-1BB transmembrane domain, and a 4-1BB intracellular domain do not negatively affect tumor specificity.

DETAILED DESCRIPTION

CAR therapy has shown significant promise to date. CD19 CARs targeting chronic lymphocytic leukemia (CLL) and more recently, acute lymphoblastic leukemia (ALL) have met with notable success. Interestingly, CARs targeting other antigens have not provided similar clinical responses. One limitation of such antigen-targeted approaches is their therapeutic applicability, which is limited only to the diseases expressing particular surface receptors and the limitations of targeting a single tumor antigen that have resulted in relapses with antigen-loss variants.

A major hurdle in tumor immunology is the induction of tumor-specific tolerance which limits the intrinsic anti-tumor efficacy of many cell based approaches. Recent studies have shown significant clinical efficacy by targeting checkpoint inhibitors leading to the approval of anti-CTLA-4 and anti-PD-1 for metastatic melanoma. In some aspects, the embodiments relate to a chimeric receptor, comprising an extracellular domain expressing of a checkpoint inhibitor and an activating intracellular domain. This has the advantage of hijacking the tolerogenic mechanisms into activating signals. This approach can be used in all clinical situations in which T cell anergy is a major aspect of the pathogenesis of the disease and where the antigen specificity is provided by the endogenous T cell repertoire.

In some aspects, the embodiments relate to a chimeric transmembrane protein, comprising an extracellular domain of an inhibitory receptor, a transmembrane domain, and an intracellular signaling domain. In some embodiments, the intracellular signaling domain can activate an immune response. The intracellular signaling domain may comprise a portion of an intracellular signaling protein. In some embodiments, the intracellular domain can be used to maintain the activation of a cell, such as a T-cell.

In some embodiments, the extracellular domain can transduce a signal to the intracellular signaling domain. For example, the extracellular domain may transduce a signal to the intracellular signaling domain upon binding an agonist of the native inhibitory receptor.

Signal transduction may comprise oligomerization of the protein. Oligomerization may comprise homo-oligomerization or hetero-oligomerization. Oligomerization may comprise dimerization of the protein, *i.e.*, homo-dimerization with a second chimeric transmembrane protein or hetero-dimerization with a different protein.

Signal transduction may comprise phosphorylation. For example, the intracellular signaling domain may comprise kinase activity and/or a phosphorylation site. Signal transduction may comprise autophosphorylation, *e.g.*, autophosphorylation of the intracellular signaling domain.

In some embodiments, the protein comprises a transmembrane domain. In some embodiments, the protein is an integral membrane protein. For example, the protein may be a type 1 membrane protein, a type 2 membrane protein, or a multi-spanning membrane protein. In some embodiments, the protein comprises the transmembrane domain of the inhibitory receptor. In some embodiments, the protein comprises the transmembrane domain of the intracellular signaling protein. The chimeric transmembrane protein may comprise a signal peptide, *e.g.*, to translocate the extracellular domain across a cell membrane. In some embodiments, the transmembrane domain comprises the sequence of IISFFLALTSTALLFLLFFLTLRFSVV (SEQ ID NO: 5). In some embodiments, the chimeric transmembrane protein comprises a signal peptide derived from CD8. In some embodiments, the signal peptide comprises the CD8 leader peptide. In some embodiments, the signal peptide comprises MALPVTALLLPLALLLHAARP (SEQ ID NO: 6).

In some embodiments, the extracellular domain is the extracellular domain of an inhibitory receptor. In some embodiments, the extracellular domain comprises a ligand-binding domain, *e.g.*, the agonist-binding domain of the inhibitory receptor. In some embodiments, the extracellular domain comprises sufficient structure to transduce a signal across the membrane in response to ligand binding. Without being bound to any particular theory, for inhibitory receptors that transduce a signal by oligomerization mediated by a multivalent ligand, the mere presence of a ligand-binding domain may be sufficient structure to transduce a signal across the membrane in response to ligand binding. Without being bound to any particular theory, for inhibitory receptors that transduce a signal by altering the orientation of a transmembrane domain relative to the cell membrane, the extracellular domain may require native structure between the ligand-binding domain and transmembrane domain to transduce a signal across the membrane in response to ligand

binding. For example, an extracellular domain may comprise the native sequence of the inhibitory receptor from its ligand-binding domain to its transmembrane domain.

The native inhibitory receptor can be a human inhibitory receptor or a mouse inhibitory receptor. Thus, the extracellular domain may comprise a human or mouse amino acid sequence. In some embodiments, the origin of the native inhibitory receptor is selected to match the species of a subject that is being treated, *e.g.*, to avoid an immune response against the chimeric transmembrane protein. Nevertheless, the native inhibitory receptor may be selected from a different species, *e.g.*, for convenience. Accordingly, the chimeric protein may or may not be xenogeneic-derived relative either to the species of cell in which the protein is expressed or the subject to which the protein is administered.

In some embodiments, the native inhibitory receptor is selected from proteins that reduce immune activity upon binding a native agonist. For example, the native inhibitory receptor may reduce T cell proliferation, T cell survival, cytokine secretion, or immune cytolytic activity upon binding a native agonist. The native inhibitory receptor may be a lymphocyte inhibitory receptor (*i.e.*, the inhibitory receptor may be expressed on lymphocytes, such as T cells). For example, the native inhibitory receptor may be expressed on T cells, and the binding of an agonist to the native inhibitory receptor may cause cell signaling that disfavors T cell proliferation, T cell survival, cytokine secretion, or immune cytolytic activity.

In some embodiments, the native inhibitory receptor may be CTLA-4 (cytotoxic T-lymphocyte-associated protein 4; CD152), PD-1 (Programmed cell death protein 1; CD279), LAG-3 (Lymphocyte-activation gene 3; CD223), or Tim-3 (T cell immunoglobulin mucin-3). Thus, in some embodiments, the extracellular domain may be the extracellular domain from CTLA-4, PD-1, LAG-3, or Tim-3. The inhibitory receptor may be PD-1. In some embodiments, the transmembrane protein comprises the extracellular domain of PD-1. In some embodiments, the sequence of the extracellular domain comprises PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQT DKLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPK AQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLV. (SEQ ID NO: 7).

In some embodiments, the intracellular signaling domain is the signaling domain of an intracellular signaling protein. In some embodiments, the intracellular signaling domain may comprise kinase activity or a phosphorylation site. The intracellular signaling domain

can, in some embodiments, activate a signaling molecule, such as a kinase or phosphorylase, *e.g.*, following signal transduction across a cell membrane. The intracellular signaling domain may signal through a downstream kinase or a phosphorylase.

The intracellular signaling protein may be a human protein or a mouse protein. Thus, the intracellular signaling domain may comprise a human or mouse amino acid sequence. In some embodiments, the intracellular signaling protein is selected to match the species of a subject and cell that is being used for treatment, *e.g.*, so that the signaling domain may utilize the cell's cytosolic machinery to activate downstream signaling molecules. Nevertheless, the intracellular signaling protein may be selected from a different species, *e.g.*, for convenience, such as described above.

In some embodiments, the intracellular signaling protein increases immune activity. Thus, signal transduction via the chimeric transmembrane protein can result in a signal cascade that increases immune activity, wherein the intracellular signaling domain mediates the intracellular signaling cascade. In some embodiments, the intracellular signaling protein can enhance T cell proliferation, T cell survival, cytokine secretion, or immune cytolytic activity. In some embodiments, the intracellular signaling protein is a transmembrane protein or the intracellular signaling protein can bind a native transmembrane protein. The intracellular signaling protein may be a lymphocyte protein (*i.e.*, the intracellular signaling protein may be expressed on lymphocytes, such as T cells).

In some embodiments, the intracellular signaling protein is CD3ζ (T-cell surface glycoprotein CD3 zeta chain; CD247), 4-1BB (tumor necrosis factor receptor superfamily member 9; CD137), or CD28 (T-cell-specific surface glycoprotein CD28; Tp44). Thus, the intracellular signaling protein may comprise a signaling domain from CD3ζ, 4-1BB, or CD28. The intracellular signaling protein may be 4-1BB. Thus, the intracellular signaling protein may comprise a signaling domain from 4-1BB. In some embodiments, the intracellular domain comprises KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO: 8).

In some embodiments, the chimeric transmembrane protein comprises a suicide domain, *i.e.*, to kill a recombinant cell comprising the protein. The suicide domain may comprise thymidine kinase activity or caspase activity. For example, the suicide domain may be a thymidine kinase or a caspase. In some embodiments, the suicide domain is the

thymidine kinase domain of HSV thymidine kinase ("HSV-TK") or the suicide domain comprises a portion of caspase 9.

In some aspects, the embodiments relates to a nucleic acid molecule encoding a chimeric transmembrane protein as described herein. The nucleic acid molecule may comprise a promoter, wherein the promoter is operably linked to a nucleotide sequence encoding the chimeric transmembrane protein, *e.g.*, for expression of a chimeric transmembrane protein in a recombinant cell. In some embodiments, the promoter is a constitutive promoter. In some embodiments, the promoter is a cell specific promoter. In some embodiments, the promoter is a tissue specific promoter.

The nucleic acid molecule may comprise the sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4. The nucleic acid molecule may comprise at least about 100, 200, 300, 400, 500, 600, or 700 consecutive nucleotides in the sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4. The nucleic acid molecule may comprise a nucleotide sequence having at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence homology with the nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4. The nucleic acid molecule may comprise a nucleotide sequence having at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence homology with at least about 100, 200, 300, 400, 500, 600, or 700 consecutive nucleotides in the nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4. For example, the nucleic acid molecule may comprise a nucleotide sequence having at least 95% sequence homology with at least 100 consecutive nucleotides in the nucleotide sequence set forth in SEQ ID NO:3.

In some embodiments, the nucleic acid molecule encodes an amino acid sequence as described herein and/or in the drawings. In some embodiments, the nucleic acid molecule encodes an amino acid sequence comprising one or more amino acid sequences set forth in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, or SEQ ID NO: 11. In some embodiments, the nucleic acid molecule may comprise a nucleotide sequence that encodes an amino acid sequence having at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence homology with a nucleotide sequence set forth herein and/or in the drawings. Homology can be identity or similarly in the context of a protein. Sequence homology may refer to sequence identity in

the context of a nucleic acid molecule. Homology can be used by employing routine tools such as Expasy, BLASTp, Clustal, and the like using default settings.

In some embodiments, the chimeric transmembrane protein comprises one or more amino acid sequences set forth in the following table:

Sequence	SEQ ID NO
IISFFLALTSTALLFLLFFLTLRFSVV	5
MALPVTALLLPLALLLHAARP	6
PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTC SFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQ PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTY LCGAISLAPKAQIKESLRAELRVTERRAEVPTAHP SPSPRPAGQFQTLV	7
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPE EEEGGCEL	8
IISFFLALTSTALLFLLFFLTLRFSVVKRGRKKLLY IFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	9
PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTC SFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQ PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTY LCGAISLAPKAQIKESLRAELRVTERRAEVPTAHP SPSPRPAGQFQTLVIISFFLALTSTALLFLLFFLTLR FSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSC RFPEEEEGGCEL	10
MALPVTALLLPLALLLHAARPPGWFLDSPDRPW NPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNW YRMSPSNQTDKLAAFPEDRSQPGQDCRFRVTQLP NGRDFHMSVVRARRNDSGTYLCGAISLAPKAQI KESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTL VIISFFLALTSTALLFLLFFLTLRFSVVKRGRKKLL YIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	11

In some embodiments, the chimeric transmembrane protein comprises an amino acid sequence having at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence homology with one of the amino acid sequences set forth herein.

Variants of the amino acid sequences described herein may be included in various embodiments. The term “variant” refers to a protein or polypeptide in which one or more

(*e.g.*, 1, 2, 3, 4, etc.) amino acid substitutions, deletions, and/or insertions are present as compared to the amino acid sequence of a protein or polypeptide, and the term includes naturally occurring allelic variants and alternative splice variants of a protein or polypeptide. The term "variant" includes the replacement of one or more amino acids in an amino acid sequence with a similar or homologous amino acid(s) or a dissimilar amino acid(s). Some variants include alanine substitutions at one or more amino acid positions in an amino acid sequence. Other substitutions include conservative substitutions that have little or no effect on the overall net charge, polarity, or hydrophobicity of the protein. Conservative substitutions may have insignificant effect on the function of the chimeric transmembrane protein. In some embodiments, the function can be the specificity of a protein when expressed in a lymphocyte, *e.g.*, a marrow-infiltrating lymphocyte (MIL), such as described in Example 3. One of skill in the art can determine if a substitution affects the function of a chimeric transmembrane protein by comparing to the sequences provided herein using a protocol identical to, or analogous to, Example 3. Non-limiting exemplary conservative substitutions are set forth in the table below. According to some embodiments, a chimeric transmembrane protein has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity with an amino acid sequence described herein.

Conservative Amino Acid Substitutions

Basic:	arginine lysine histidine
Acidic:	glutamic acid aspartic acid
Uncharged Polar:	glutamine asparagine serine threonine tyrosine
Non-Polar:	phenylalanine tryptophan cysteine

	glycine alanine valine proline methionine leucine isoleucine

The table below sets out another scheme of conservative amino acid substitutions.

Original Residue	**Conservative Substitutions**
Ala	Gly; Ser; Thr
Arg	Lys; Gln
Asn	Gln; His; Ser
Asp	Glu; Asn
Cys	Ser
Gln	Asn; Ser; Asp; Glu
Glu	Asp; Gln; Lys
Gly	Ala; Pro; Asn
His	Asn; Gln; Tyr
Ile	Leu; Val; Met; Val; Phe
Leu	Ile; Val; Met; Phe
Lys	Arg; Gln
Met	Leu; Tyr; Ile; Val; Phe
Pro	Ser; Thr; Ala; Gly
Phe	Met; Leu; Tyr; Trp
Ser	Thr; Gly; Asn; Asp
Thr	Ser; Asn
Trp	Tyr; Phe
Tyr	Trp; Phe
Val	Ile; Leu; Met; Phe

Accordingly, in some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 of the amino acid residues of an amino acid sequence disclosed herein are modified with conservative substitutions. In

some embodiments, only 1, 2, 3, 4 or 5 amino acid residues are substituted with conservative substitutions.

In some embodiments, the chimeric transmembrane protein comprises a sequence of SEQ ID NO: 10 or SEQ ID NO: 11 or a variant thereof. SEQ ID NO: 10 is a combination of SEQ ID NO: 5, 7, and 8. SEQ ID NO: 11 is a combination of SEQ ID NO: 5, 6, 7, and 8. In some embodiments, the sequence of SEQ ID NO: 6 is replaced with another signal peptide or leader sequence, that can assist in trafficking the chimeric transmembrane protein to the extracellular membrane. In some embodiments, the transmembrane domain, *e.g.*, SEQ ID NO: 5, is replaced with a different transmembrane protein. In some embodiments, the transmembrane domain is the transmembrane domain of PD-1. In some embodiments, the transmembrane domain is the transmembrane domain of 4-1BB.

In some aspects, the embodiments relate to a recombinant cell, comprising a nucleic acid as disclosed herein. In some embodiments, the embodiments relate to a recombinant cell, comprising a chimeric transmembrane protein as described herein. In some embodiments, the cell comprises a chimeric protein comprising a protein of SEQ ID NO: 5, 6, 7, 8, 9, 10, or 11 or a variant thereof. In some embodiments, the cell is a lymphocyte. The cell may be a T cell. In some embodiments, the cell may be a tumor-infiltrating lymphocyte ("TIL") or a marrow infiltrating lymphocyte ("MIL").

In some embodiments, the cell comprising a chimeric transmembrane protein described herein persist longer and/or remain in an active state longer in a subject when administered to the subject as compared to a cell without a chimeric transmembrane protein.

In some aspects, the embodiments relate to a method for making a recombinant cell, comprising transfecting a cell with a nucleic acid molecule as described herein. In some aspects, the embodiments relate to a method for making a recombinant cell, comprising transfecting a cell with a nucleic acid molecule encoding an amino acid sequence as described herein. The nucleic acid molecule may be a plasmid. The cell can be transfected by a plasmid comprising one or more nucleotide sequences as described herein. The cell can also be infected with a virus or virus-like particle comprising the nucleic acid molecule. In some embodiments, the cell is a TIL or a MIL. In some embodiments, the MIL is an activated MIL. MILs can be activated, for example, by incubating them with anti-CD3/anti-CD28 beads and appropriate cytokines, *e.g.*, under hypoxic conditions. An example of growing the MILs under hypoxic conditions can found, for example, in

WO2016037054, which is hereby incorporated by reference in its entirety. In some embodiments, the nucleic acid molecule is transfected into a cell after the cell has been incubated in a hypoxic environment as described herein. In some embodiments, the nucleic acid molecule is transfected into a cell after the cell has been incubated in a hypoxic environment for about 1, 2, 3, 4, or 5 days. In some embodiments, the cell is then incubated under normoxic conditions for about 1, 2, 3, 4, or 5 days.

In some embodiments, a MIL comprising the chimeric transmembrane protein is prepared according to a method described in WO2016037054, which is hereby incorporated by reference in its entirety. In some embodiments, the method may comprise removing cells in the bone marrow, lymphocytes, and/or marrow infiltrating lymphocytes ("MILs") from the subject; incubating the cells in a hypoxic environment, thereby producing activated MILs; and administering the activated MILs to the subject. The cells can also be activated in the presence of anti-CD3/anti-CD28 antibodies and cytokines as described herein. A nucleic acid molecule encoding a chimeric transmembrane protein, such as one of those described herein, can be transfected or infected into a cell before or after the MIL is incubated in a hypoxic environment.

The hypoxic environment may comprise less than about 21 % oxygen, such as less than about 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 1 1%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, or less than about 3% oxygen. For example, the hypoxic environment may comprise about 0% oxygen to about 20% oxygen, such as about 0% oxygen to about 19% oxygen, about 0% oxygen to about 18% oxygen, about 0% oxygen to about 17% oxygen, about 0% oxygen to about 16% oxygen, about 0% oxygen to about 15% oxygen, about 0% oxygen to about 14% oxygen, about 0% oxygen to about 13% oxygen, about 0% oxygen to about 12% oxygen, about 0% oxygen to about 11% oxygen, about 0% oxygen to about 10% oxygen, about 0% oxygen to about 9% oxygen, about 0% oxygen to about 8% oxygen, about 0% oxygen to about 7% oxygen, about 0% oxygen to about 6% oxygen, about 0% oxygen to about 5% oxygen, about 0% oxygen to about 4% oxygen, or about 0% oxygen to about 3% oxygen. In some embodiments, the hypoxic environment comprises about 1 % to about 7% oxygen. In some embodiments, the hypoxic environment is about 1% to about 2% oxygen. In some embodiments, the hypoxic environment is about 0.5% to about 1.5% oxygen. In some embodiments, the hypoxic environment is about 0.5% to about 2% oxygen. The hypoxic environment may comprise about 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 1 1%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or about 0% oxygen.

In some embodiments, the hypoxic environment comprises about 7%, 6%, 5%, 4%, 3%, 2%, or 1% oxygen.

Incubating MILs in a hypoxic environment may comprise incubating the MILs, *e.g.*, in tissue culture medium, for at least about 1 hour, such as at least about 12 hours, 18 hours, 24 hours, 30 hours, 36 hours, 42 hours, 48 hours, 60 hours, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 1 1 days, 12 days, 13 days, or even at least about 14 days. Incubating may comprise incubating the MILs for about 1 hour to about 30 days, such as about 1 day to about 20 days, about 1 day to about 14 days, or about 1 day to about 12 days. In some embodiments, incubating MILs in a hypoxic environment comprises incubating the MILs in a hypoxic environment for about 2 days to about 5 days. The method may comprise incubating MILs in a hypoxic environment for about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 day, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the method comprises incubating the MILs in a hypoxic environment for about 3 days. In some embodiments, the method comprises incubating the MILs in a hypoxic environment for about 2 days to about 4 days. In some embodiments, the method comprises incubating the MILs in a hypoxic environment for about 3 days to about 4 days.

In some embodiments, the method further comprises incubating the MILs in a normoxic environment, *e.g.*, after incubating the MILs in a hypoxic environment.

The normoxic environment may comprise at least about 21% oxygen. The normoxic environment may comprise about 5% oxygen to about 30% oxygen, such as about 10% oxygen to about 30% oxygen, about 15% oxygen to about 25% oxygen, about 18% oxygen to about 24% oxygen, about 19% oxygen to about 23% oxygen, or about 20% oxygen to about 22% oxygen. In some embodiments, the normoxic environment comprises about 21 % oxygen.

Incubating MILs in a normoxic environment may comprise incubating the MILs, *e.g.*, in tissue culture medium, for at least about 1 hour, such as at least about 12 hours, 18 hours, 24 hours, 30 hours, 36 hours, 42 hours, 48 hours, 60 hours, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 1 1 days, 12 days, 13 days, or even at least about 14 days. Incubating may comprise incubating the MILs for about 1 hour to about 30 days, such as about 1 day to about 20 days, about 1 day to about 14 days, about 1 day to about 12 days, or about 2 days to about 12 days.

In some embodiments, the cell is transfected or infected with a nucleic acid molecule encoding a chimeric transmembrane protein described herein after being placed in a normoxic environment or before it is placed in a normoxic environment.

In some embodiments, the MILs are obtained by extracting a bone marrow sample from a subject and culturing/incubating the cells as described herein. In some embodiments, the bone marrow sample is centrifuged to remove red blood cells. In some embodiments, the bone marrow sample is not subject to apheresis. In some embodiments, the bone marrow sample does not comprise peripheral blood lymphocytes ("PBL") or the bone marrow sample is substantially free of PBLs. These methods select for cells that are not the same as what have become to be known as TILs. Thus, a MIL is not a TIL. TILs can be selected by known methods to one of skill in the art and can be transfected or infected with the nucleic acid molecules described herein such that the TILs can express the chimeric transmembrane protein described herein.

In some embodiments, the cells are also activated by culturing with antibodies to CD3 and CD28. This can be performed, for example by incubating the cells with anti-CD3/anti-CD28 beads that are commercially available or that can be made by one of skill in the art. The cells can then be plated in a plate, flask, or bag. Hypoxic conditions can be achieved by flushing either the hypoxic chamber or cell culture bag for 3 minutes with a 95% Nitrogen and 5% CO_2 gas mixture. This can lead to, for example, 1-2% or less O_2 gas in the receptacle. Cells can be then cultured as described herein or as in the examples of WO2016037054, which is hereby incorporated by reference.

In some embodiments, a hypoxic MIL comprising a chimeric transmembrane protein as described herein is provided. In some embodiments, the hypoxic MIL is in an environment of about 0.5% to about 5% oxygen gas. In some embodiments, the hypoxic MIL is in an environment of about 1% to about 2% oxygen gas. In some embodiments, the hypoxic MIL is in an environment of about 1% to about 3% oxygen gas. In some embodiments, the hypoxic MIL is in an environment of about 1% to about 4% oxygen gas. A hypoxic MIL is a MIL that has been incubated in a hypoxic environment, such as those described herein, for a period of time, such as those described herein. Without being bound to any particular theory, a hypoxic MIL will undergo changes in protein and/or gene expression that affect the anti-tumor capabilities of the MIL. As described herein, the hypoxic MIL can also be activated with the presence of anti-CD3/anti-CD28 beads or other similar activating reagents. Thus, a hypoxic MIL can also be an activated-hypoxic MIL.

In some aspects, the embodiments relates to a method for increasing an immune response in a subject, comprising administering to the subject a recombinant cell as described herein. In some embodiments, the embodiments relate to a method for treating a neoplasm in a subject, comprising administering to the subject a recombinant cell as described herein. The neoplasm may be a benign neoplasm, a malignant neoplasm, or a secondary neoplasm. The neoplasm may be cancer. The neoplasm may be a lymphoma or a leukemia, such as chronic lymphocytic leukemia ("CLL") or acute lymphoblastic leukemia ("ALL"). The neoplasm may be multiple myeloma as well as any solid tumor (*e.g.*, breast cancer, prostate cancer, lung cancer, esophageal cancer, brain cancer, kidney cancer, bladder cancer, pancreatic cancer, osteosarcoma, and the like).

The method may comprise administering to the subject a plurality of recombinant cells as described herein. The method may comprise administering to the subject an effective amount of recombinant cells as described herein.

In some embodiments, the cell is obtained from the subject. The cell that is transfected or infected may be obtained from the subject. The cell can be obtained as described herein. For example, a cell that is administered may be autologous to the subject. In some embodiments, the cell that is administered is allogeneic to the subject. The cell may be obtained from the subject and transfected or infected with a nucleic acid encoding a chimeric transmembrane protein as described herein. The cell may be a daughter cell, wherein a parent of the daughter cell was obtained from the subject. The recombinant cell may have been transfected or infected with the nucleic acid or a parent of the recombinant cell may have been transfected or infected with the nucleic acid. In some embodiments, the cell after being transfected or infected expresses a protein comprising one or more of the amino sequences described herein.

The method may further comprise making the recombinant cell, wherein making the recombinant cell comprises transfecting or infecting a cell with a nucleic acid encoding a chimeric transmembrane protein, such as those described herein. In some embodiments, the chimeric transmembrane protein comprises an amino acid sequence set forth in any one of SEQ ID NO: 5, 6, 7, 8, 9, 10, or 11 or a variant thereof. Similarly, the method may further comprise making a plurality of recombinant cells, wherein making the plurality of recombinant cells comprises transfecting or infecting a plurality of cells with nucleic acids encoding a chimeric transmembrane protein, such as those described herein. The method may further comprise expanding a parent cell, *e.g.*, the recombinant cell may be a daughter

cell of the parent cell. The method may comprise expanding a population of cells, *e.g.*, the method may comprise administering to the subject a plurality of recombinant cells as described herein, and each cell of the plurality of recombinant cells may be a daughter cell of a parent cell.

The method may further comprise isolating the cell or a parent cell from the subject.

The method may further comprise sorting the cell, *e.g.*, by fluorescence activated cell sorting ("FACS") or magnetic activated cell sorting ("MACS").

The cells can be administered to a subject by any suitable route in, for example, a pharmaceutically acceptable composition. In some embodiments, the composition is pyrogen free. For example, administration of the cells may be carried out using any method known in the art. For example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intracerebroventricular, or intrathecal. For parenteral administration, the cells may be administered by either intravenous, subcutaneous, or intramuscular injection, in compositions with pharmaceutically acceptable vehicles or carriers. The cells can be formulated for parenteral administration by injection, for example, by bolus injection or continuous infusion. The compositions can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents, for example, suspending, stabilizing, and/or dispersing agents.

For administration by injection, it can be desired to use the cells in solution in a sterile aqueous vehicle which may also contain other solutes such as buffers or preservatives as well as sufficient quantities of pharmaceutically acceptable salts or of glucose to make the solution isotonic. In some embodiments, the pharmaceutical compositions may be formulated with a pharmaceutically acceptable carrier to provide sterile solutions or suspensions for injectable administration. In particular, injectables can be prepared in conventional forms, either as liquid solutions or suspensions or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, or the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. Suitable pharmaceutical carriers are described in "Remington's pharmaceutical Sciences" by E. W. Martin.

The subject may be any organism that comprises immune cells. For example, the subject may be selected from rodents, canines, felines, porcines, ovines, bovines, equines, and primates. The subject may be a mouse or a human.

The subject may have a neoplasm. The neoplasm may be a benign neoplasm, a malignant neoplasm, or a secondary neoplasm. The neoplasm may be cancer. The neoplasm may be a lymphoma or a leukemia, such as chronic lymphocytic leukemia ("CLL") or acute lymphoblastic leukemia ("ALL"). The subject may have a glioblastoma, medulloblastoma, breast cancer, head and neck cancer, kidney cancer, ovarian cancer, Kaposi's sarcoma, acute myelogenous leukemia, and B-lineage malignancies. The subject may have multiple myeloma.

In some embodiments, the subject is a subject "in need thereof." As used herein, the phrase "in need thereof" means that the subject has been identified or suspected as having a need for the particular method or treatment. In some embodiments, the identification can be by any means of diagnosis. In any of the methods and treatments described herein, the subject can be in need thereof.

As used herein, terms such as "a," "an," and "the" include singular and plural referents unless the context clearly demands otherwise.

As used in this document, terms "comprise," "have," "has," and "include" and their conjugates, as used herein, mean "including but not limited to." While various compositions, and methods are described in terms of "comprising" various components or steps (interpreted as meaning "including, but not limited to"), the compositions, methods, and devices can also "consist essentially of" or "consist of" the various components and steps, and such terminology should be interpreted as defining essentially closed-member groups.

As used herein, the terms "treat," "treated," or "treating" mean both therapeutic treatment wherein the object is to slow down (lessen) an undesired physiological condition, disorder or disease, or obtain beneficial or desired clinical results. For purposes of the embodiments described herein, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms; diminishment of extent of condition, disorder or disease; stabilized (*i.e.*, not worsening) state of condition, disorder or disease; delay in onset or slowing of condition, disorder or disease progression; amelioration of the condition, disorder or disease state or remission (whether partial or total), whether detectable or undetectable; an amelioration of at least one measurable physical parameter, not necessarily

discernible by the patient; or enhancement or improvement of condition, disorder or disease. Thus, "treatment of cancer" or "treating cancer" means an activity that alleviates or ameliorates any of the primary phenomena or secondary symptoms associated with the cancer or any other condition described herein. In some embodiments, the cancer that is being treated is one of the cancers recited herein.

EXAMPLES

The following examples are illustrative, but not limiting, of the methods and compositions described herein. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in therapy and that are obvious to those skilled in the art are within the spirit and scope of the embodiments.

Example 1: CAR Transduction Protocol

16-24 hours prior to transduction, T-cells were plated in an appropriate media and were stimulated with CD3, CD28 and IL-2. The cells were then placed in an incubator (37°C / 5% CO_2) overnight. After 16-24 hours, as much media as possible was removed without disturbing the cells. The CAR virus was then added to the cells and placed back in the incubator for 4-12 hours. After 4-12 hours, the appropriate volume of media containing IL-2 was added back to the cells and then placed back in the incubator. Cells were left in the incubator to grow, splitting and changing media when necessary, for 3-12 days. CAR transduction may be checked by a variety of methods including, but not limited to flow cytometry, western blotting or fluorescence microscopy, if a fluorescent reporter gene has been used.

Example 2: CAR Transfection Protocol

293T cells were passaged every two days in DMEM + 10% FBS for at least three passages at a cell density at which they never became more than 80% confluent. One day prior to transfection, the 293T cells were seeded at a density at which they were about 80% confluent after 24 hours (on the day of transfection). On the day of transfection, media was removed and enough fresh media was added to cover the cells. In a separate tube, VSV-G, Gag, Pol & Rev plasmids, a transfection reagent and the CAR plasmid were combined and incubated at room temperature for 10-20 minutes. This mixture was then added drop-wise to the 293T cells and incubated overnight. 12-24 hours after transfection, the media was either completely changed or additional fresh media was added. At both 48hrs and 72hrs post-transfection, virus-containing media from the cells was collected and cells were replenished with fresh media. Any cells in the collected media were removed by

centrifugation or filtration. The collected media was then spun in an ultracentrifuge to pellet the virus. Excess media was removed and the virus was re-suspended in DMEM or HBSS, aliquoted into sterile tubes and stored at -80°C until used.

EXAMPLE 3: MIL FUNCTION AND GROWTH IS NOT NEGATIVELY AFFECTED BY THE PRESENCE OF A CHIMERIC RECEPTOR PROTEIN.

MILs obtained from subjects were activated and expanded as described herein. Briefly, after the marrow sample was obtained from the subject, the cells were incubated under hypoxic conditions in the presence of anti-CD3/anti-CD28 beads and cytokines as described in WO2016037054, which is hereby incorporated by reference. The MILs were then infected with a virus comprising a nucleic acid molecule encoding a chimeric transmembrane protein comprising SEQ ID NO: 11. The cells were then grown under normoxic conditions and allowed to expand. The control and infected MILs were contacted with different cell types. Neither the expansion of the MILS nor the ability of the MILs to recognize antigens was negatively affected by the presence of the chimeric transmembrane protein. These results demonstrate that adding a chimeric transmembrane protein to a MIL is not detrimental to its functions and growth. The results are illustrated in Figure 6, Panel A and B, which are from two different patients.

In summary, the embodiments and examples provided herein demonstrate that cells expressing a chimeric transmembrane protein can be effectively used to treat cancer and/or modulate an immune response.

Any U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications, including CAS numbers, referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety.

What is claimed is:

1. A chimeric transmembrane protein, comprising:

the extracellular domain of an inhibitory receptor; and

an intracellular signaling domain that can activate an immune response, wherein the intracellular signaling domain comprises a portion of an intracellular signaling protein.

2. The protein of claim 1, wherein the protein comprises a sequence of SEQ ID NO: 10.

3. The protein of claim 2, wherein the protein comprises a sequence of SEQ ID NO: 11.

4. The protein of claim 1, wherein the protein comprises a sequence of SEQ ID NO: 7 and SEQ ID NO: 8.

5. The protein of claim 1, wherein the protein comprises a sequence of SEQ ID NO: 9

6. The protein of any one of the preceding claims, wherein the intracellular signaling domain comprises kinase activity.

7. The protein of any one of the preceding claims, wherein the intracellular signaling domain comprises a phosphorylation site.

8. The protein of any one of the preceding claims, wherein the protein comprises the transmembrane domain of the inhibitory receptor or the transmembrane domain of the intracellular signaling protein.

9. The protein of any one of the preceding claims, wherein the inhibitory receptor is a human inhibitory receptor or a mouse inhibitory receptor.

10. The protein of any one of the preceding claims, wherein the inhibitory receptor reduces immune activity upon binding a native agonist.

11. The protein of any one of the preceding claims, wherein the inhibitory receptor can reduce T cell proliferation, T cell survival, cytokine secretion, or immune cytolytic activity upon binding a native agonist.

12. The protein of any one of the preceding claims, wherein the inhibitory receptor is a lymphocyte inhibitory receptor.

13. The protein of claim 12, wherein the inhibitory receptor is CTLA-4, PD-1, LAG-3, or Tim-3.

14. The protein of claim 13, wherein the inhibitory receptor is PD-1.

15. The protein of any one of the preceding claims, wherein the intracellular signaling protein is a human protein or a mouse protein.

16. The protein of any one of the preceding claims, wherein the intracellular signaling protein increases immune activity.

17. The protein of any one of the preceding claims, wherein the intracellular signaling protein can enhance T cell proliferation, T cell survival, cytokine secretion, or immune cytolytic activity.

18. The protein of any one of the preceding claims, wherein the intracellular signaling protein is a transmembrane protein or the intracellular signaling protein can bind a native transmembrane protein.

19. The protein of any one of the preceding claims, wherein the intracellular signaling protein is a lymphocyte protein.

20. The protein of claim 19, wherein the intracellular signaling protein is CD3ζ, 4-1BB, or CD28.

21. The protein of claim 19, wherein the intracellular signaling protein is 4-1BB.

22. The protein of any one of the preceding claims, further comprising a suicide domain.

23. The protein of claim 22, wherein the suicide domain has thymidine kinase activity or the suicide domain is a caspase.

24. The protein of claim 23, wherein the suicide domain is the thymidine kinase domain of HSV thymidine kinase or the suicide domain comprises a portion of caspase 9.

25. A nucleic acid encoding the chimeric transmembrane protein of any one of claims 1-24.

26. A recombinant cell, comprising the nucleic acid of claim 25.

27. A recombinant cell, comprising the chimeric transmembrane protein of any one of claims 1-24.

28. The cell of claim 26 or 27, wherein the cell is a lymphocyte.

29. The cell of claim 27, wherein the cell is a T cell.

30. The cell of claim 27, wherein the cell is a tumor infiltrating lymphocyte ("TIL").

31 The cell of claim 27, wherein the cell is a marrow infiltrating lymphocyte ("MIL").

32. The cell of claim 31, wherein the MIL is a hypoxic MIL.

33. A method for making a recombinant cell, comprising transfecting or infecting a cell with the nucleic acid molecule encoding a chimeric transmembrane protein of any one of claims 1-24.

34. The method of claim 33, wherein the cell is a MIL.

35. The method of claims 33 or 34 further comprising incubating the MIL under hypoxic conditions prior to transfecting or infecting the cell with the nucleic acid molecule encoding the chimeric transmembrane protein.

36. The method of claim 35, wherein the hypoxic conditions comprise about 0.5% to about 5% oxygen gas.

37. The method of claim 35, wherein the hypoxic conditions comprise about 1% to about 2% oxygen gas.

38. The method of any one of claims 35-37, further comprising incubating the cells under normoxic conditions after the hypoxic incubation.

39. The method of any one of claims 33 to 38, further comprising contacting the cell with anti-CD3/anti-CD28 beads.

40. A method for increasing an immune response in a subject, comprising administering to the subject the recombinant cell of any one of claims 26-32.

41. The method of claim 40, further comprising making the recombinant cell, wherein making the recombinant cell comprises transfecting a cell with a nucleic acid encoding the chimeric transmembrane protein.

42. The method of claim 40, further comprising isolating the cell from the subject.

43. The method of claim 40, wherein the subject has a neoplasm.

44. The method of claim 43, wherein the neoplasm is a leukemia, lymphoma, or multiple myeloma.

45. The method of claim 40, wherein the subject is a human.

46. A method for treating a neoplasm in a subject, comprising administering to the subject the recombinant cell of any one of claims 26-32.

47. The method of claim 46, further comprising making the recombinant cell, wherein making the recombinant cell comprises transfecting a cell with a nucleic acid encoding the chimeric transmembrane protein.

48. The method of claim 46, further comprising isolating the cell from the subject.

49. The method of claim 46, wherein the subject has a neoplasm.

50. The method of claim 49, wherein the neoplasm is a multiple myeloma, leukemia or lymphoma.

51. The method of claim 46, wherein the subject is a human.

52. The method of claim 46, further comprising prior to administering the cell to the subject:

contacting the cell with anti-CD3/anti-CD28 beads;
incubating the cell under hypoxic conditions; and
incubating the cell under normoxic conditions.

53. The method of claim 52, wherein the cell is incubated under hypoxic conditions for about 0.5 to about 4 days.

54. The method of claim 52, wherein the cell is incubated under normoxic conditions for about 0.5 to about 4 days.

Figure 1

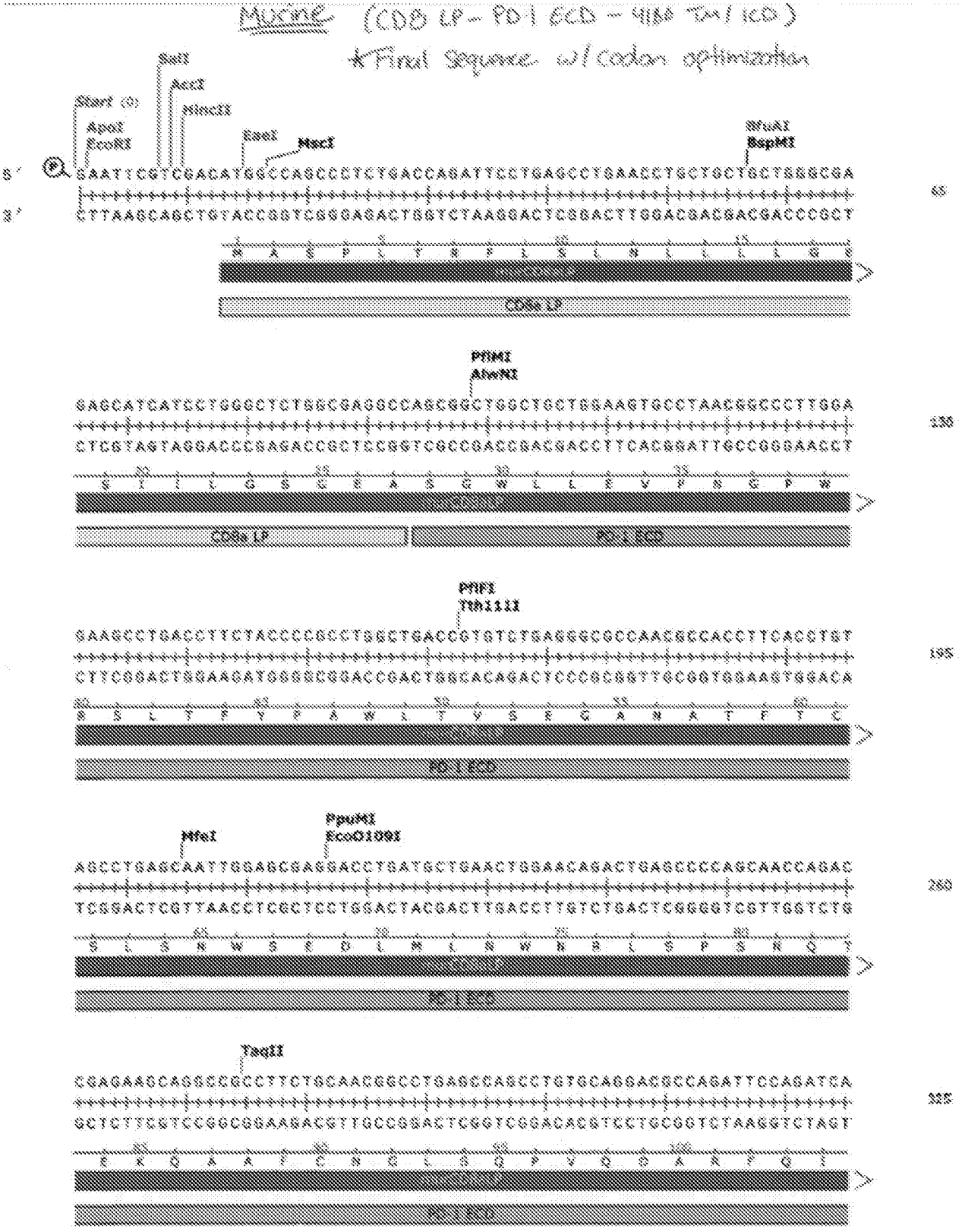

Murine (CD8 LP - PD1 ECD - 4188 Tm/ICD)
*Final Sequence w/ codon optimization
SalI
AccI
HincII
Start (R)
ApaI
EcoRI
EagI
MscI
BfuAI
BspMI
5'
3'
GAATTCGTCGACATGGCCAGCCCTCTGACCAGATTCCTGAGCCTGAACCTGCTGCTGCTGGGCGA
CTTAAGCAGCTGTACCGGTCGGGAGACTGGTCTAAGGACTCGGACTTGGACGACGACGACCCGCT
60
CD8α LP
PflMI
AlwNI
GAGCATCATCCTGGGCTCTGGCGAGGCCAGCGGCTGGCTGCTGGAAGTGCCTAACGGCCCTTGGA
CTCGTAGTAGGACCCGAGACCGCTCCGGTCGCCGACCGACGACCTTCACGGATTGCCGGGAACCT
120
CD8α LP
PD-1 ECD
PflFI
TthIIII
GAAGCCTGACCTTCTACCCCGCCTGGCTGACCGTGTCTGAGGGCGCCAACGCCACCTTCACCTGT
CTTCGGACTGGAAGATGGGGCGGACCGACTGGCACAGACTCCCGCGGTTGCGGTGGAAGTGGACA
180
PD-1 ECD
MfeI
PpuMI
EcoO109I
AGCCTGAGCAATTGGAGCGAGGACCTGATGCTGAACTGGAACAGACTGAGCCCCAGCAACCAGAC
TCGGACTCGTTAACCTCGCTCCTGGACTACGACTTGACCTTGTCTGACTCGGGGTCGTTGGTCTG
240
PD-1 ECD
TaqII
CGAGAAGCAGGCGGCCTTCTGCAACGGCCTGAGCCAGCCTGTGCAGGACGCCAGATTCCAGATCA
GCTCTTCGTCCGGCGGAAGACGTTGCCGGACTCGGTCGGACACGTCCTGCGGTCTAAGGTCTAGT
300
PD-1 ECD

Figure 1 (continued)

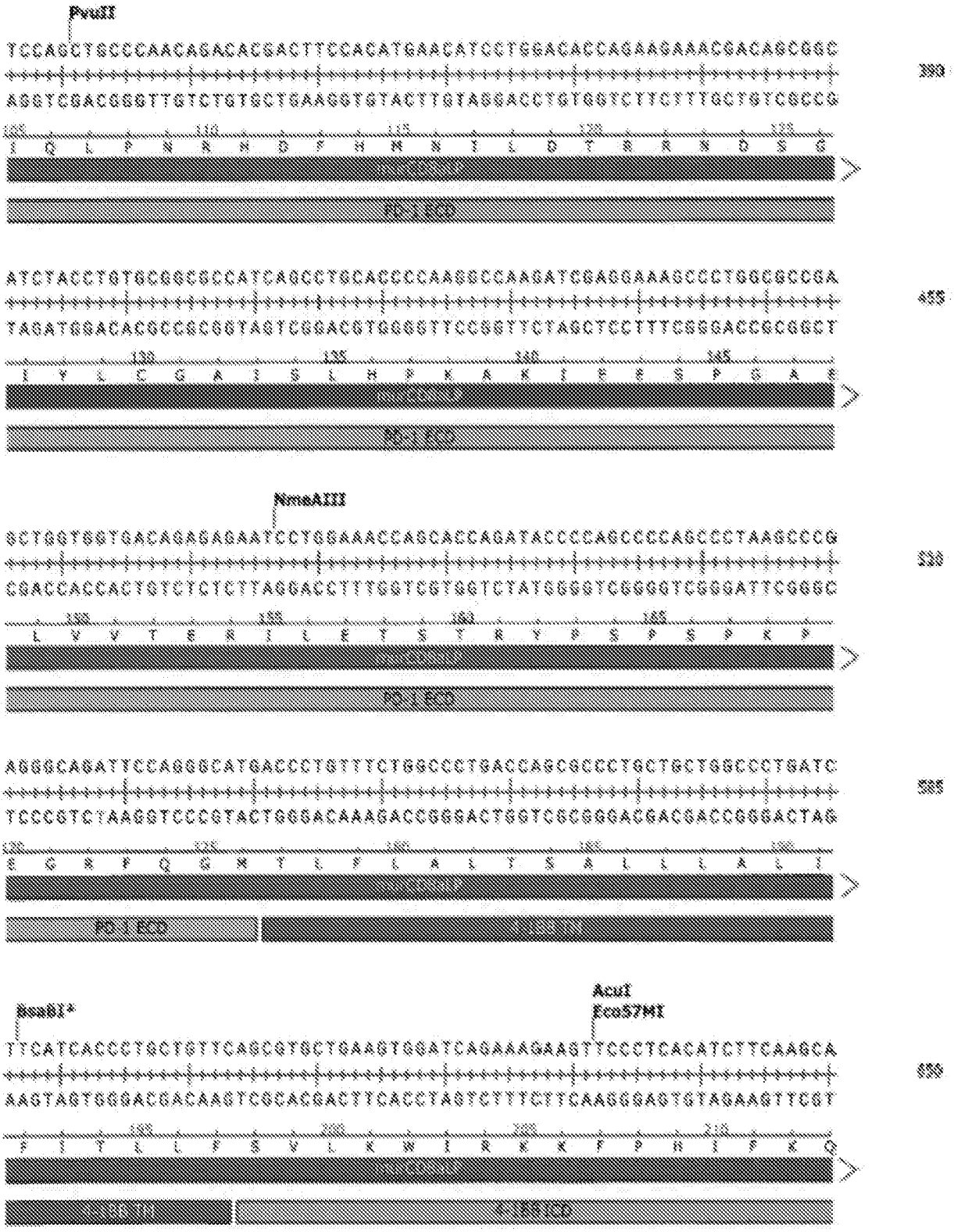
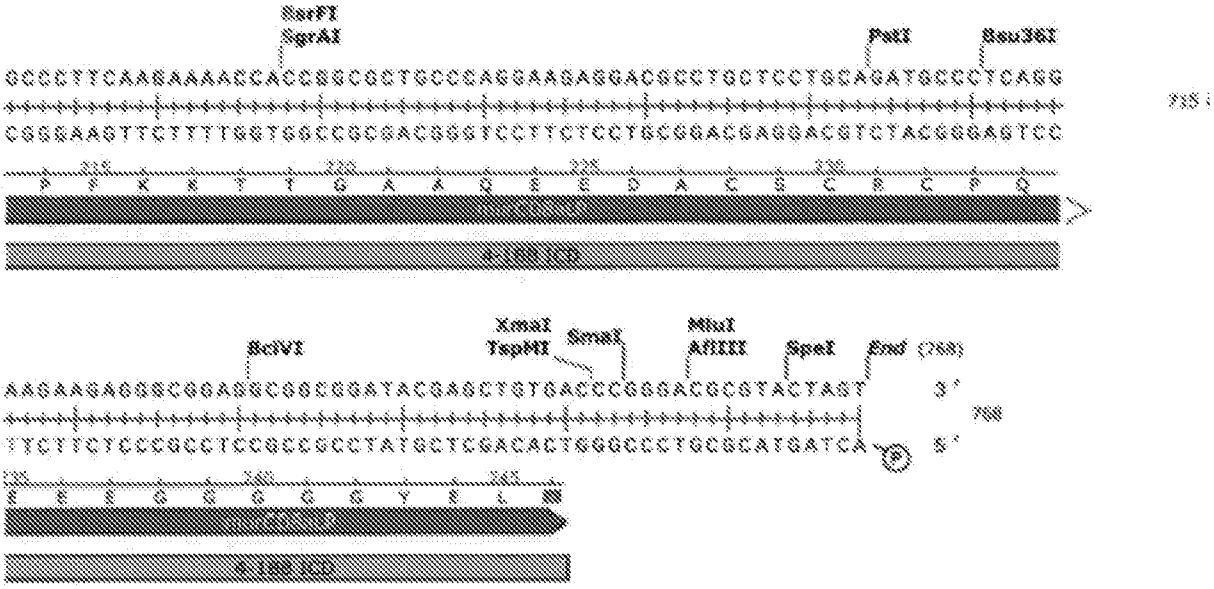


Figure 1 (continued)



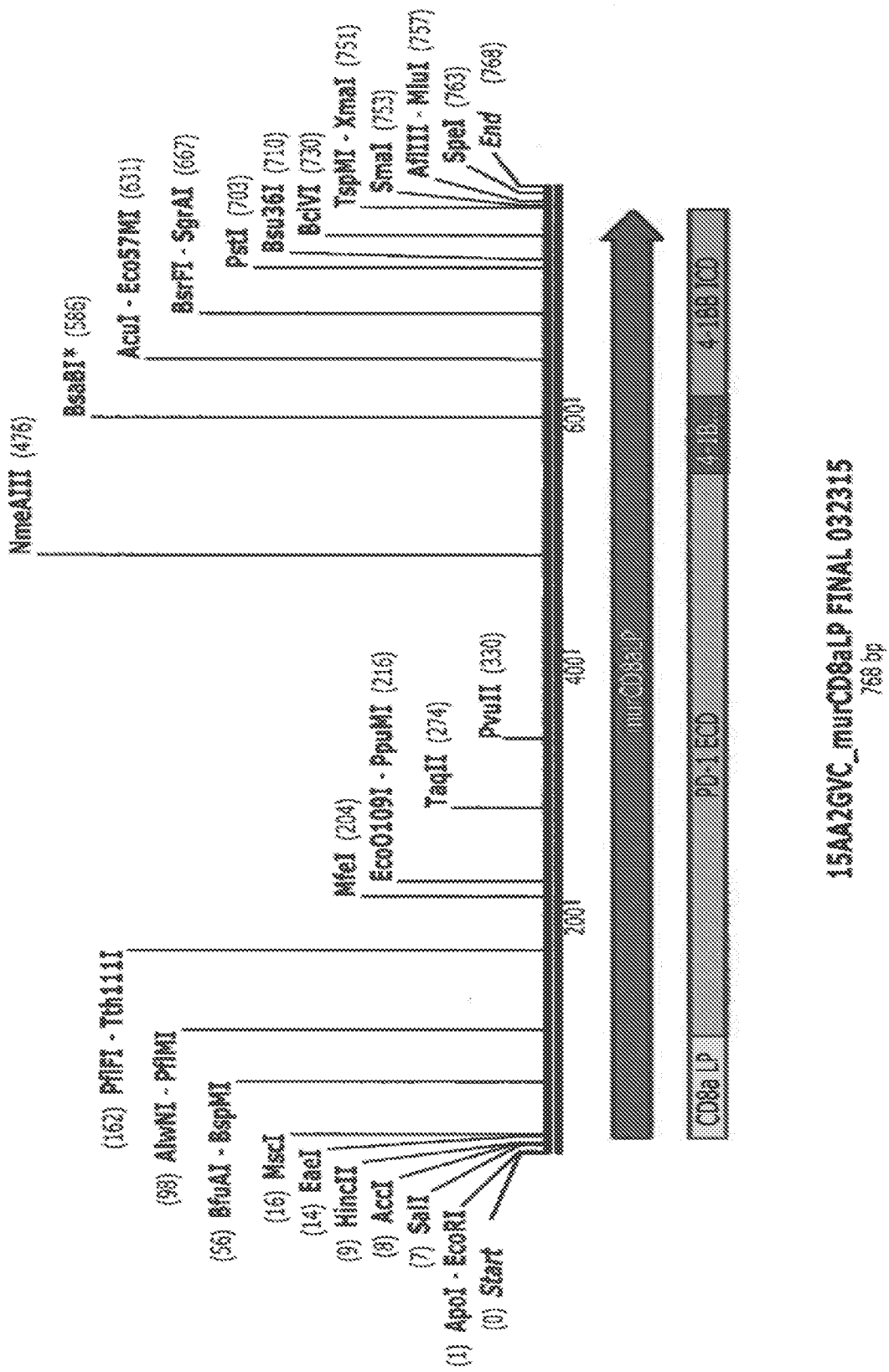


Figure 1 (cont.)

Figure 2

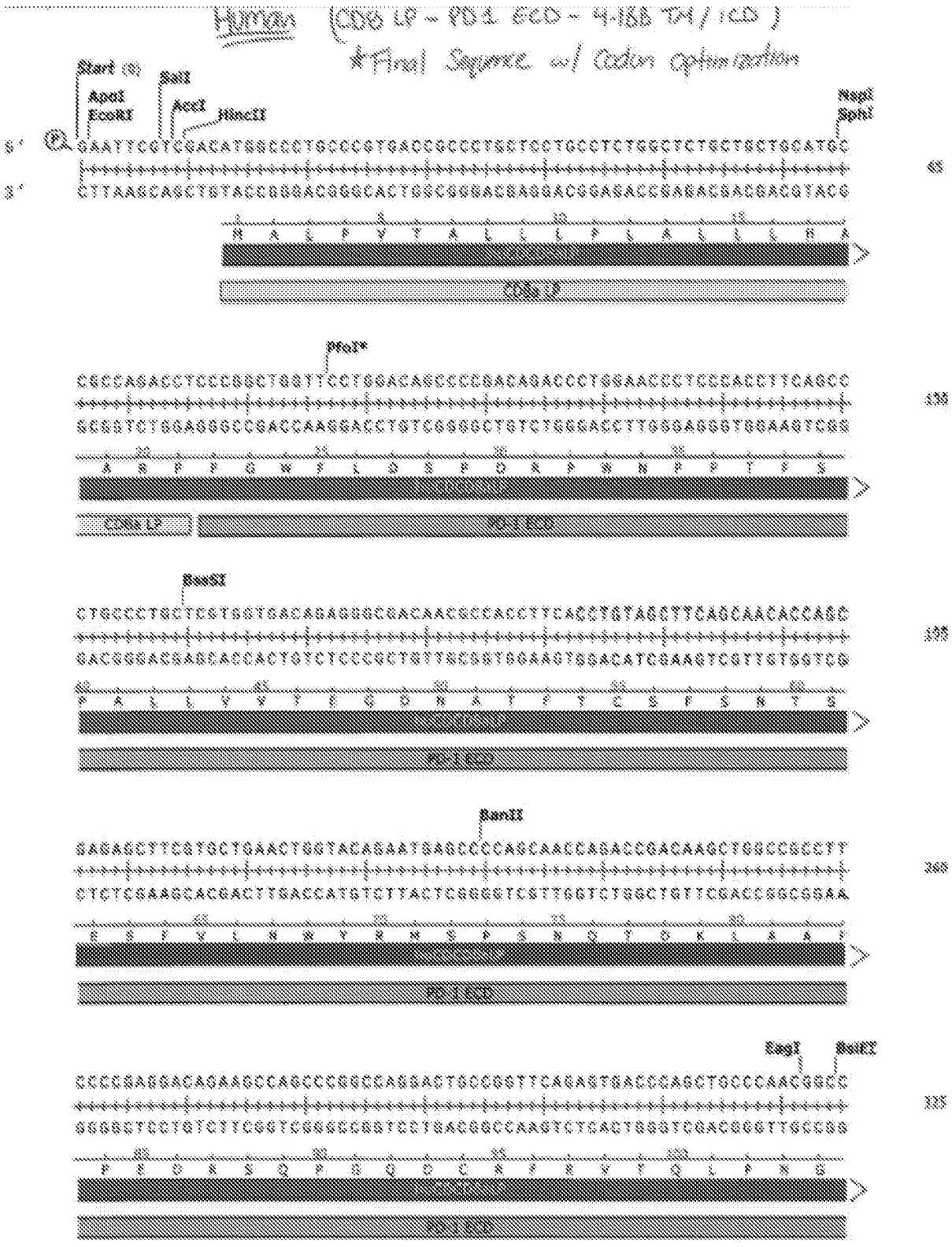

Human (CD8 LP - PD-L ECD - 4-1BB TM / ICD)
*Final Sequence w/ Codon optimization
Start (G)
ApaI
EcoRI
SalI
AccI
HincII
NaeI
SphI
PstI*
BamHI
BanII
EagI
BstEII
CD8α LP
PD-L ECD
4-1BB TM/ICD

Figure 2 (continued)

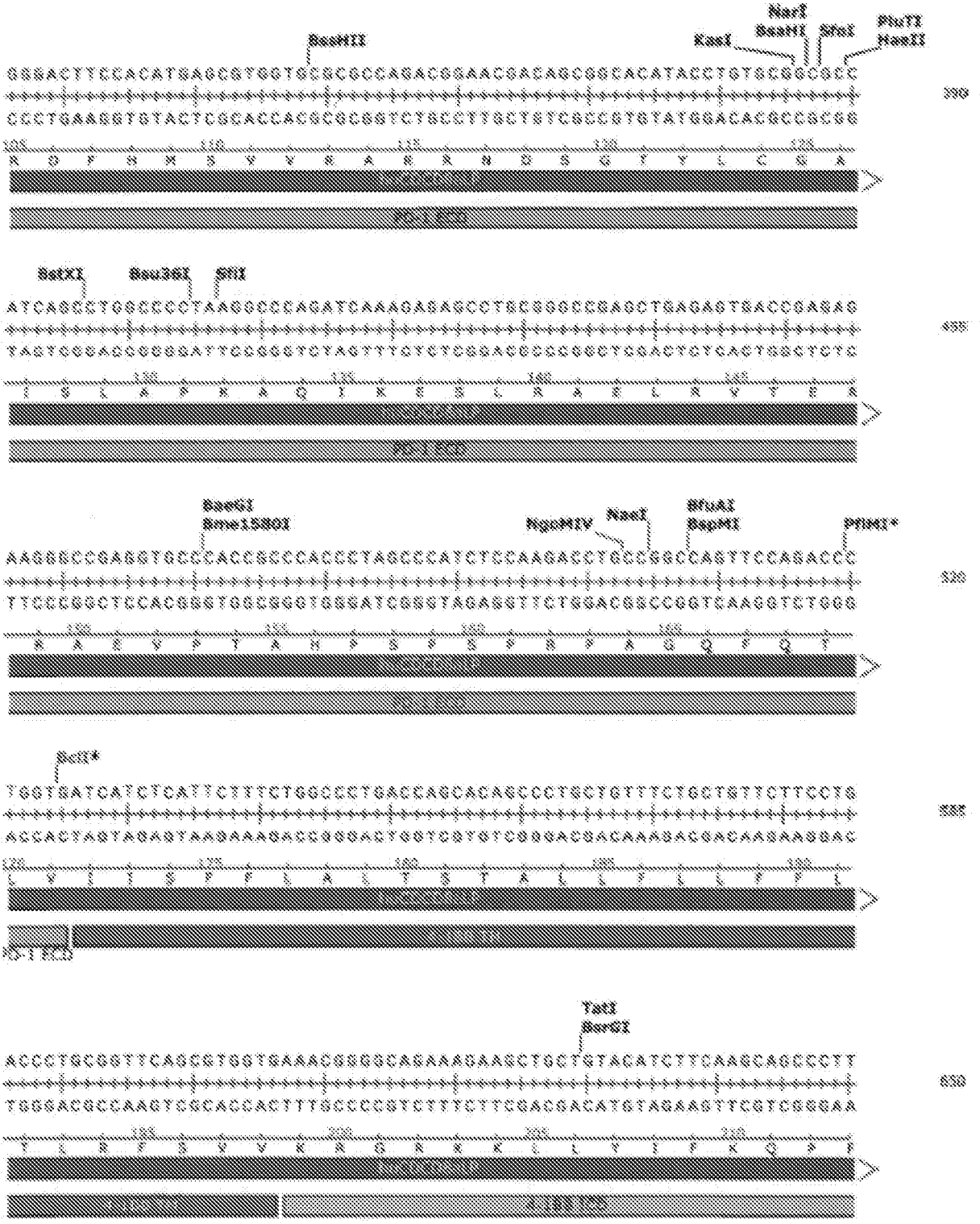
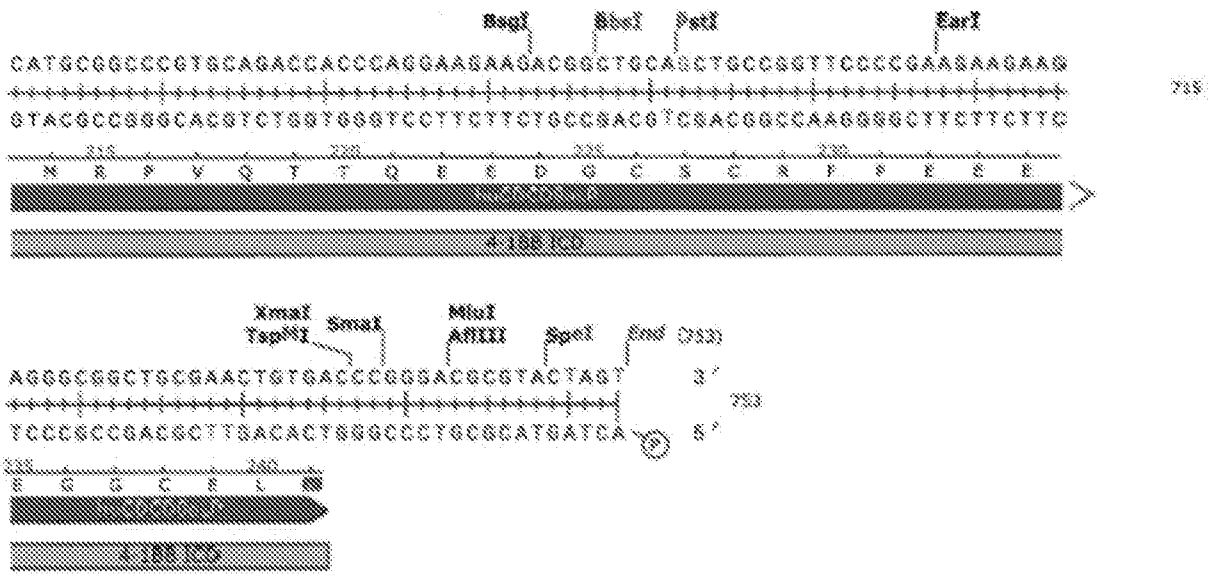


Figure 2 (continued)



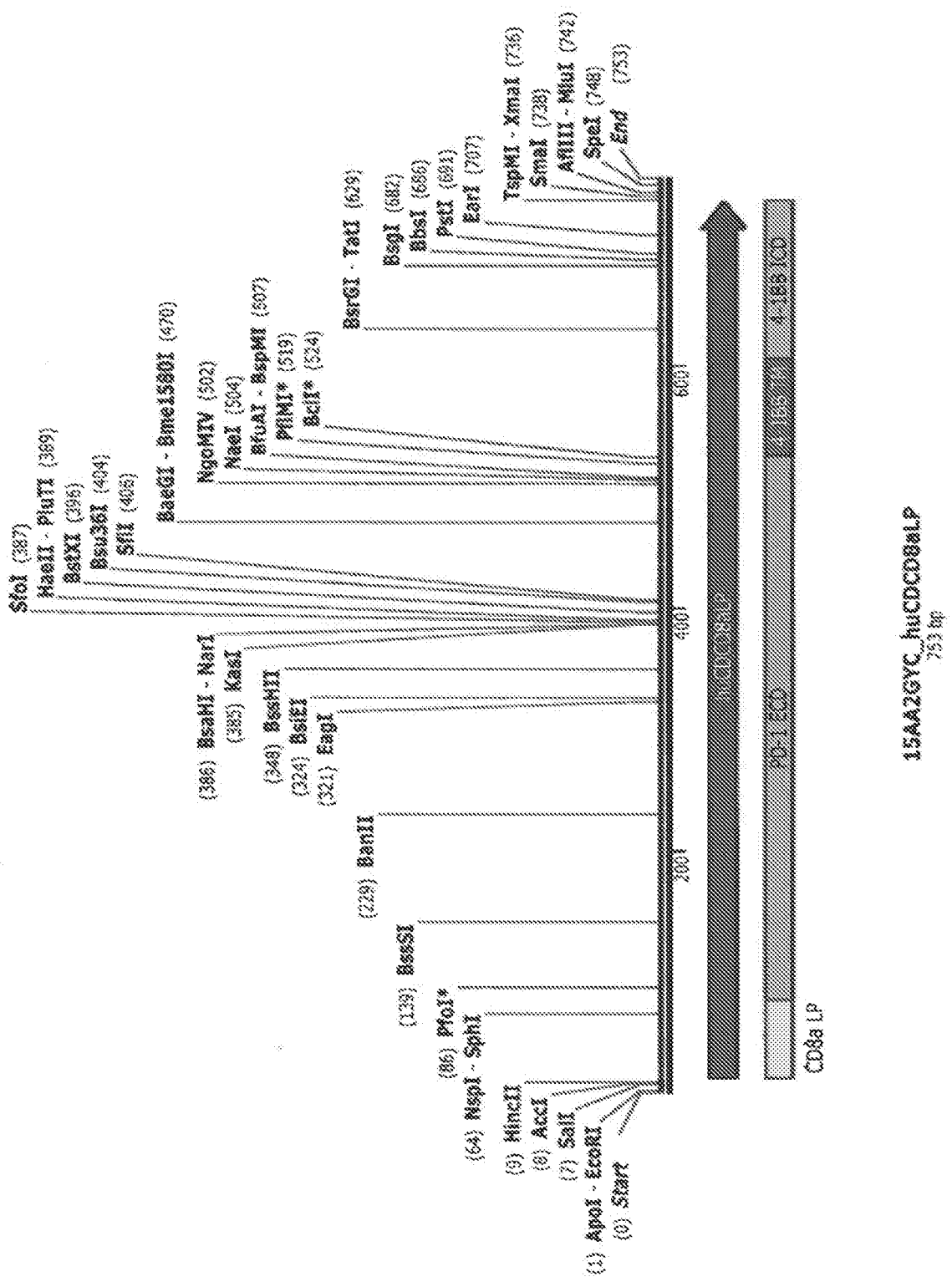


Figure 2 (cont.)

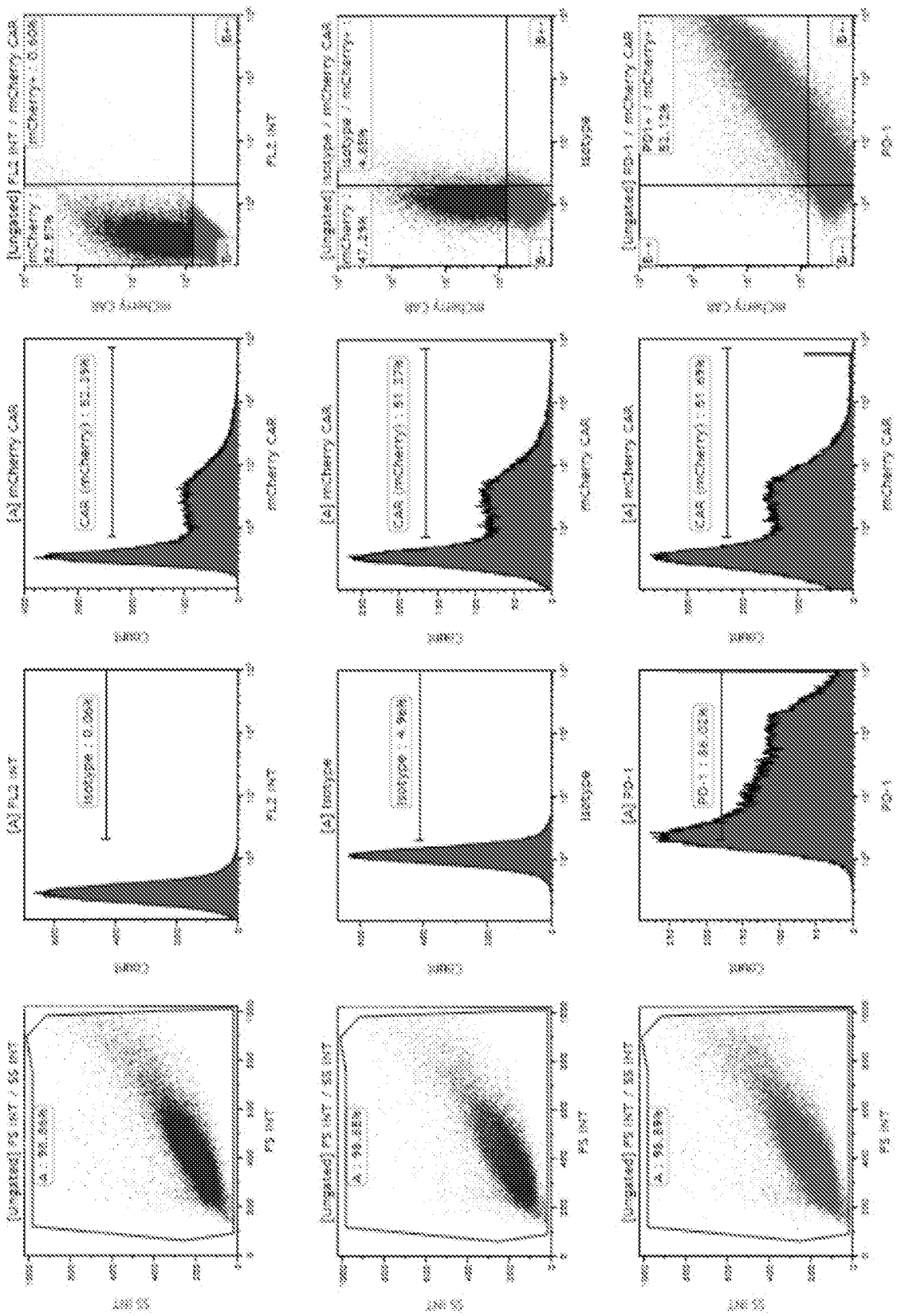


Figure 3

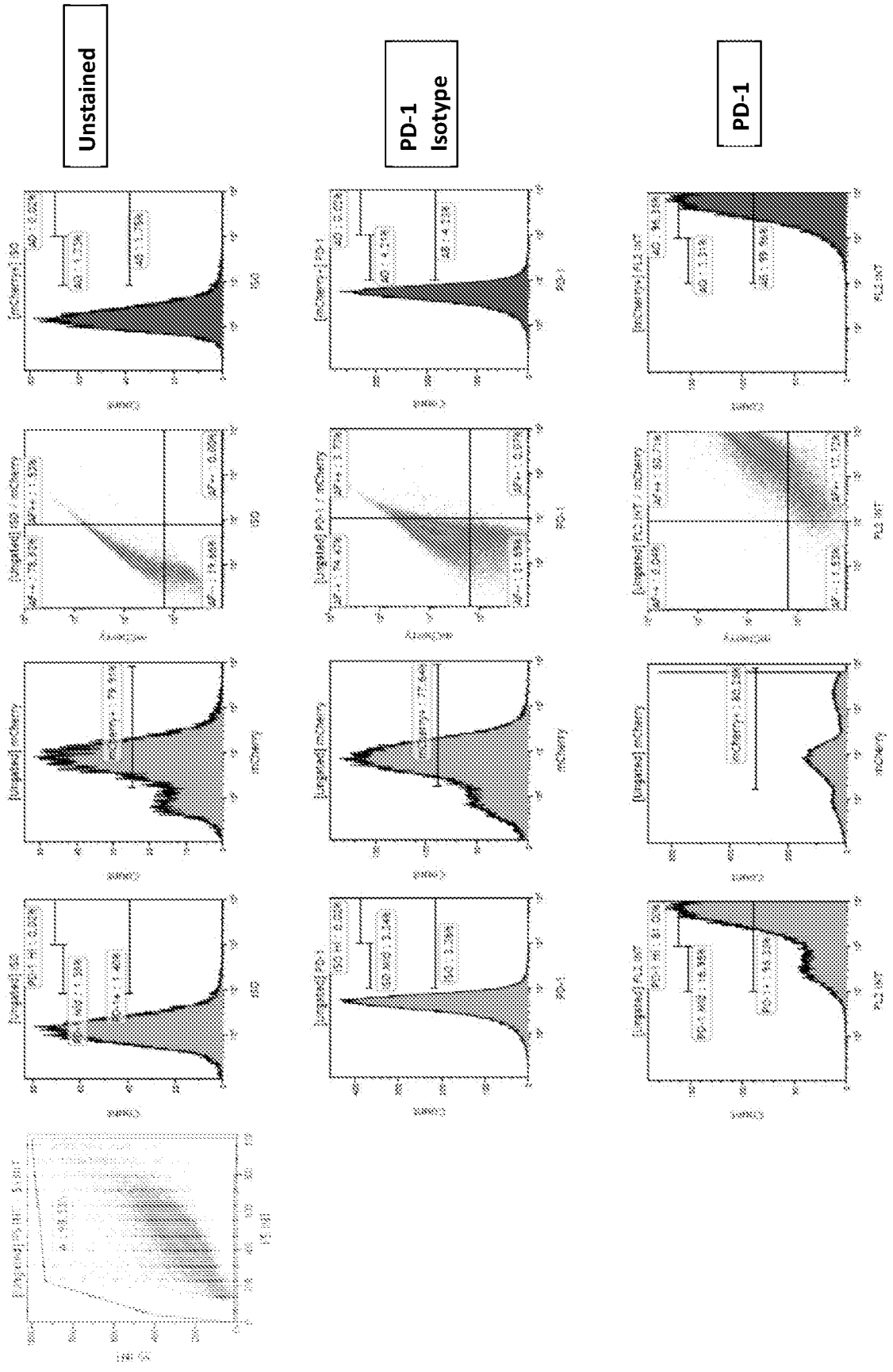


Figure 4

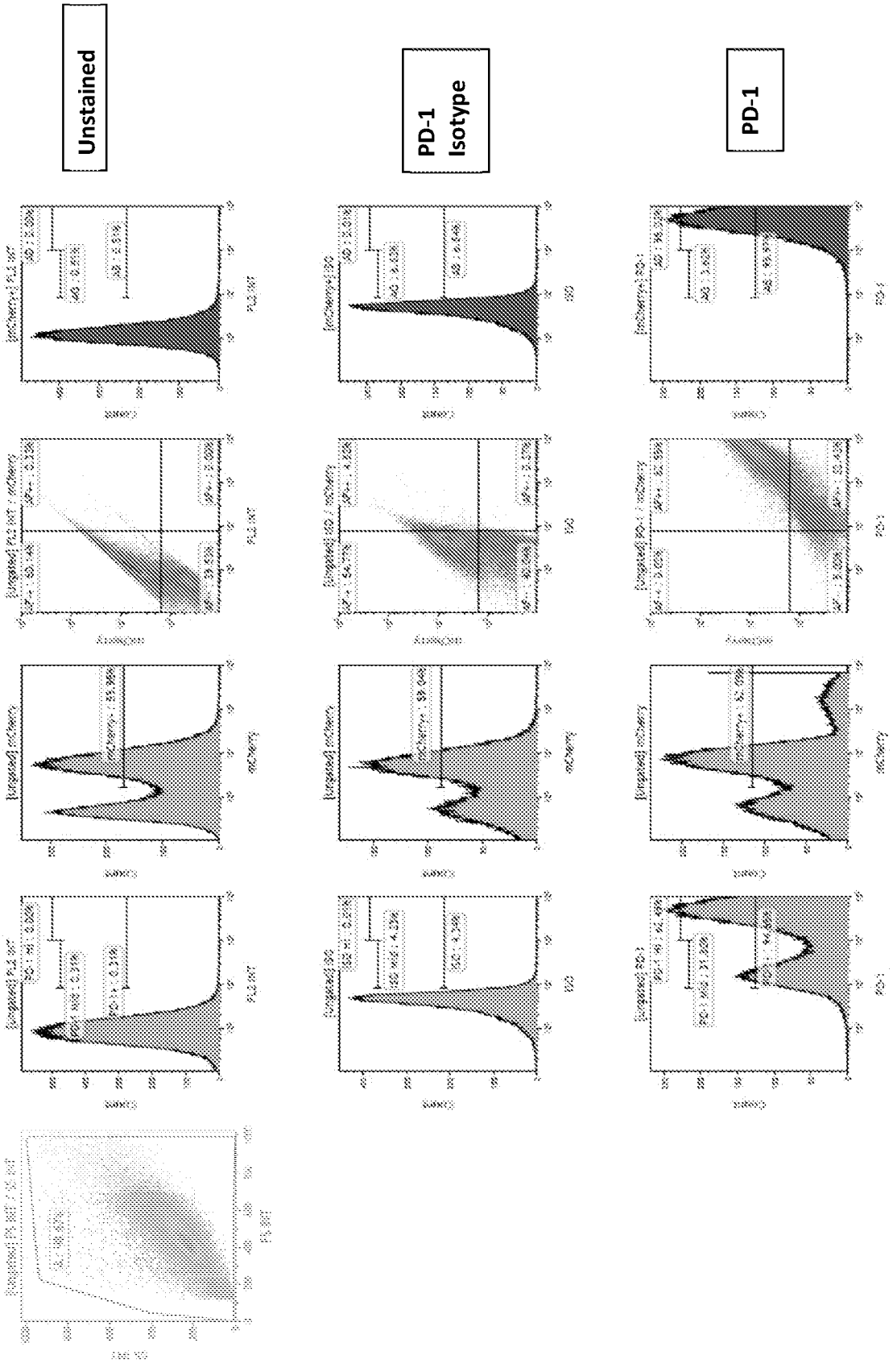
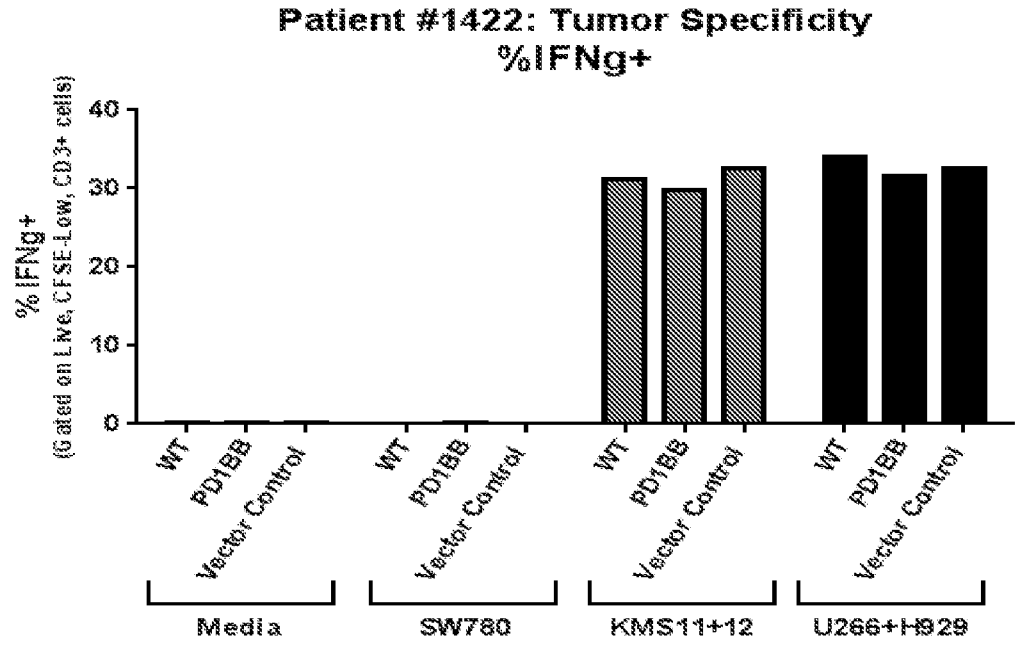


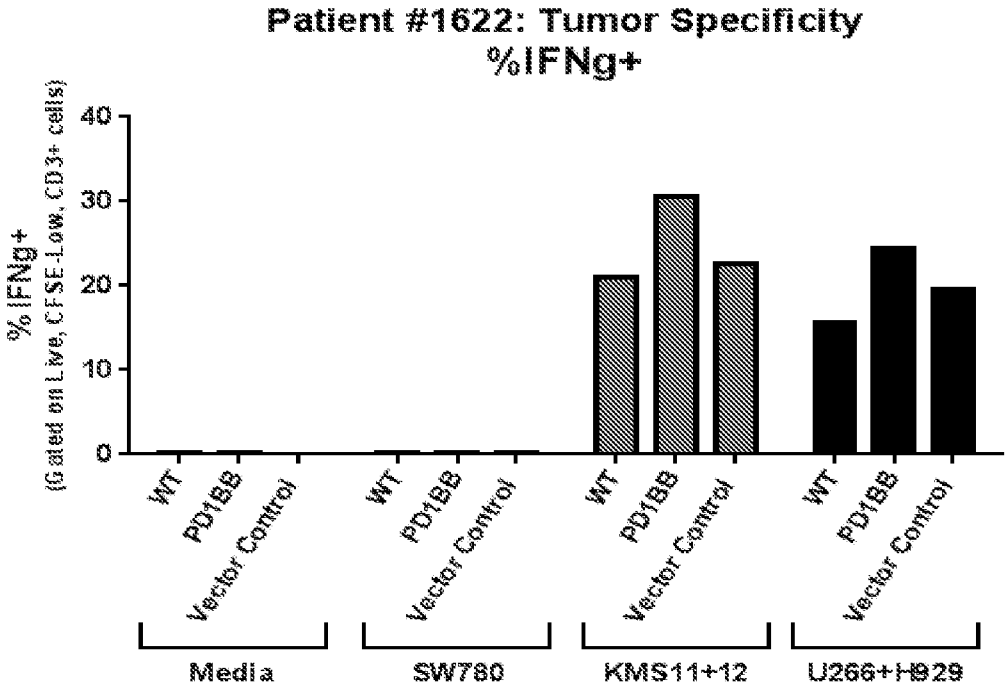
Figure 5

Figure 6

A.


Patient #1422: Tumor Specificity
%IFNg+
% IFNg+
(Gated on Live, CFSE-Low, CD3+ cells)
40
30
20
10
0
WT
PD1BB
Vector Control
Media
SW780
KMS11+12
U266+H929

B.


Patient #1622: Tumor Specificity
%IFNg+
% IFNg+
(Gated on Live, CFSE-Low, CD3+ cells)
40
30
20
10
0
WT
PD1BB
Vector Control
Media
SW780
KMS11+12
U266+H929

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/040010

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2016.01) C07K 19/00, C12N 15/62, C07K 14/705, C07K 14/725, A61K 38/17, A61P 35/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (2016.01) C07K, C12N, A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013019615 A2 UNIV PENNSYLVANIA]; JUNE CARL H; ZHAO YANGBING 07 Feb 2013 (2013/02/07) the wole document	1-29,33,34,40-51
Y		30-32,35-39,52-54
X	US 2014242049 A1 NAT CANCER CT 28 Aug 2014 (2014/08/28) the whole document	1-29,33,34,40-51
Y		30-32,35-39,52-54
X	SHIN, Jae Hun, et al. Positive conversion of negative signaling of CTLA4 potentiates antitumor efficacy of adoptive T-cell therapy in murine tumor models. Blood, 2012, 119.24: 5678-5687. doi:10.1182/blood-2011-09-380519 Retrieved from the internet: < http://www.bloodjournal.org/content/119/24/5678.full> 14 Jun 2012 (2012/06/14) the whole document	1-29,33,34,40-51

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
12 Sep 2016	22 Sep 2016
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616	Authorized officer HOROWITZ Anat Telephone No. 972-2-5651689

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/040010

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y		30-32,35-39,52-54
Y	WO 2014100385 A1 ANTHROGENESIS CORP?[US] 26 Jun 2014 (2014/06/26) [06][0138]	30
Y	NOONAN, Kimberly A., et al. Adoptive transfer of activated marrow-infiltrating lymphocytes induces measurable antitumor immunity in the bone marrow in multiple myeloma. Science translational medicine, 2015, 7.288: 288ra78-288ra78 Retrieved from the internet: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4634889/> DOI: 10.1126/scitranslmed.aaa7014 20 May 2015 (2015/05/20) the whole document	31,32
P,Y	WO 2016037054 A1 UNIV JOHNS HOPKINS 10 Mar 2016 (2016/03/10) the wole document	35-39,52-54

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/040010

B. FIELDS SEARCHED:

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

Databases consulted: THOMSON INNOVATION, CAPLUS, BIOSIS, EMBASE, MEDLINE, Google Scholar
Search terms used: Chimeric antigen receptor, fusion, PD-1, programmed cell death protein 1, CD279, CTLA-4, cytotoxic T lymphocyte associated protein 4, CD152, LAG-3, Lymphocyte-activation gene 3, CD223, Tim-3, T cell immunoglobulin mucin-3
AND CD3 zeta or T cell surface glycoprotein CD3 zeta or CD247 or 4-1BB or tumor necrosis factor receptor superfamily

member 9 or CD137 or CD28 or Tp44

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2016/040010

Patent document cited search report	Publication date	Patent family member(s)	Publication Date
WO 2013019615 A2	07 Feb 2013	WO 2013019615 A2	07 Feb 2013
		WO 2013019615 A3	08 May 2014
		AU 2012290342 A1	30 Jan 2014
		CA 2842368 A1	07 Feb 2013
		CL 2014000195 A1	08 Aug 2014
		CN 104114233 A	22 Oct 2014
		CO 6862107 A2	10 Feb 2014
		EA 201490364 A1	29 Aug 2014
		EC SP14013186 A	31 Mar 2014
		EP 2736540 A2	04 Jun 2014
		EP 2736540 A4	29 Apr 2015
		GT 201400015 A	16 Dec 2014
		IL 230496 D0	31 Mar 2014
		JP 2014524234 A	22 Sep 2014
		KR 20140045533 A	16 Apr 2014
		MA 35360 B1	01 Aug 2014
		MX 2014001222 A	15 Sep 2014
		PE 15202014 A1	17 Nov 2014
		US 2014219975 A1	07 Aug 2014
US 2014242049 A1	28 Aug 2014	US 2014242049 A1	28 Aug 2014
		JP 2014532642 A	08 Dec 2014
		KR 20130045824 A	06 May 2013
		KR 101471647 B1	11 Dec 2014
		WO 2013062365 A2	02 May 2013
		WO 2013062365 A3	20 Jun 2013
WO 2016037054 A1	10 Mar 2016	WO 2016037054 A1	10 Mar 2016
WO 2014100385 A1	26 Jun 2014	WO 2014100385 A1	26 Jun 2014
		AU 2013204922 A1	10 Jul 2014

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2016/040010

Patent document cited search report	Publication date	Patent family member(s)	Publication Date
		AU 2013204922 B2	14 May 2015
		CA 2895840 A1	26 Jun 2014
		CN 105246912 A	13 Jan 2016
		EP 2935321 A1	28 Oct 2015
		EP 2935321 A4	06 Jul 2016
		HK 1215267 A1	19 Aug 2016
		IL 239500 D0	31 Aug 2015
		JP 2016507499 A	10 Mar 2016
		KR 20150099576 A	31 Aug 2015
		SG 11201504855W A	30 Jul 2015
		US 2015307623 A1	29 Oct 2015

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 108137707 A
(43)申请公布日 2018.06.08

(21)申请号 201680048831.3

(22)申请日 2016.06.29

(30)优先权数据
62/186108 2015.06.29 US

(85)PCT国际申请进入国家阶段日
2018.02.23

(86)PCT国际申请的申请数据
PCT/US2016/040010 2016.06.29

(87)PCT国际申请的公布数据
WO2017/004150 EN 2017.01.05

(71)申请人 约翰霍普金斯大学
地址 美国马里兰州

(72)发明人 I.M.博雷洛 S.李 K.A.努南 D.M.帕多尔

(74)专利代理机构 中国专利代理(香港)有限公司 72001
代理人 段菊兰 杨思捷

(51)Int.Cl.
C07K 19/00(2006.01)
C12N 15/62(2006.01)
C07K 14/705(2006.01)
C07K 14/725(2006.01)
A61K 38/17(2006.01)
A61P 35/00(2006.01)

权利要求书2页 说明书12页 附图12页

(54)发明名称

免疫检查点嵌合受体疗法

(57)摘要

在一些方面,该实施方案涉及与嵌合跨膜蛋白相关的组合物和方法。该嵌合跨膜蛋白可以包含抑制性受体的胞外域和可以激活免疫反应的胞内信号传导域。

CN 108137707 A

1.嵌合跨膜蛋白,包含:

抑制性受体的胞外域;和

可以激活免疫反应的胞内信号传导域,其中所述胞内信号传导域包含胞内信号蛋白的一部分。

2.权利要求1的蛋白,其中所述蛋白包含SEQ ID NO:10的序列。

3.权利要求2的蛋白,其中所述蛋白包含SEQ ID NO:11的序列。

4.权利要求1的蛋白,其中所述蛋白包含SEQ ID NO:7和SEQ ID NO:8的序列。

5.权利要求1的蛋白,其中所述蛋白包含SEQ ID NO:9的序列。

6.前述权利要求任一项的蛋白,其中所述胞内信号传导域包含激酶活性。

7.前述权利要求任一项的蛋白,其中所述胞内信号传导域包含磷酸化位点。

8.前述权利要求任一项的蛋白,其中所述蛋白包含所述抑制性受体的跨膜结构域或所述胞内信号蛋白的跨膜结构域。

9.前述权利要求任一项的蛋白,其中所述抑制性受体是人抑制性受体或小鼠抑制性受体。

10.前述权利要求任一项的蛋白,其中所述抑制性受体在结合到天然激动剂时降低免疫活性。

11.前述权利要求任一项的蛋白,其中所述抑制性受体在结合到天然激动剂时可以降低T细胞增殖、T细胞存活、细胞因子分泌或免疫溶细胞活性。

12.前述权利要求任一项的蛋白,其中所述抑制性受体是淋巴细胞抑制性受体。

13.权利要求12的蛋白,其中所述抑制性受体是CTLA-4、PD-1、LAG-3或Tim-3。

14.权利要求13的蛋白,其中所述抑制性受体是PD-1。

15.前述权利要求任一项的蛋白,其中所述胞内信号蛋白是人蛋白或小鼠蛋白。

16.前述权利要求任一项的蛋白,其中所述胞内信号蛋白提高免疫活性。

17.前述权利要求任一项的蛋白,其中所述胞内信号蛋白可以增强T细胞增殖、T细胞存活、细胞因子分泌或免疫溶细胞活性。

18.前述权利要求任一项的蛋白,其中所述胞内信号蛋白是跨膜蛋白,或所述胞内信号蛋白可以结合天然跨膜蛋白。

19.前述权利要求任一项的蛋白,其中所述胞内信号蛋白是淋巴细胞蛋白。

20.权利要求19的蛋白,其中所述胞内信号蛋白是CD3ζ、4-1BB或CD28。

21.权利要求19的蛋白,其中所述胞内信号蛋白是4-1BB。

22.前述权利要求任一项的蛋白,进一步包含自杀结构域。

23.权利要求22的蛋白,其中所述自杀结构域具有胸苷激酶活性,或所述自杀结构域是半胱天冬酶。

24.权利要求23的蛋白,其中所述自杀结构域是HSV胸苷激酶的胸苷激酶结构域,或所述自杀结构域包含半胱天冬酶9的一部分。

25.核酸,其编码权利要求1-24任一项的嵌合跨膜蛋白。

26.重组细胞,其包含权利要求25的核酸。

27.重组细胞,其包含权利要求1-24任一项的嵌合跨膜蛋白。

28.权利要求26或27的细胞,其中所述细胞是淋巴细胞。

29.权利要求27的细胞，其中所述细胞是T细胞。

30.权利要求27的细胞，其中所述细胞是肿瘤浸润淋巴细胞(“TIL”)。

31.权利要求27的细胞，其中所述细胞是骨髓浸润淋巴细胞(“MIL”)。

32.权利要求31的细胞，其中所述MIL是低氧MIL。

33.制造重组细胞的方法，包括用编码权利要求1-24任一项的嵌合跨膜蛋白的核酸分子转染或感染细胞。

34.权利要求33的方法，其中所述细胞是MIL。

35.权利要求33或34的方法，进一步包括在用编码所述嵌合跨膜蛋白的核酸分子转染或感染所述细胞之前在低氧条件下孵育所述MIL。

36.权利要求35的方法，其中所述低氧条件包含大约0.5％至大约5％的氧气。

37.权利要求35的方法，其中所述低氧条件包含大约1％至大约2％的氧气。

38.权利要求35-37任一项的方法，进一步包括在低氧孵育之后在常氧条件下孵育所述细胞。

39.权利要求33至38任一项的方法，进一步包括使所述细胞与抗CD3/抗CD28珠粒接触。

40.在受试者中提高免疫反应的方法，包括向所述受试者施用权利要求26-32任一项的重组细胞。

41.权利要求40的方法，进一步包括制造所述重组细胞，其中制造所述重组细胞包括用编码所述嵌合跨膜蛋白的核酸转染细胞。

42.权利要求40的方法，进一步包括从受试者分离所述细胞。

43.权利要求40的方法，其中所述受试者患有肿瘤。

44.权利要求43的方法，其中所述肿瘤是白血病、淋巴瘤或多发性骨髓瘤。

45.权利要求40的方法，其中所述受试者是人类。

46.在受试者中治疗肿瘤的方法，包括向所述受试者施用权利要求26-32任一项的重组细胞。

47.权利要求46的方法，进一步包括制造所述重组细胞，其中制造所述重组细胞包括用编码所述嵌合跨膜蛋白的核酸转染细胞。

48.权利要求46的方法，进一步包括从受试者分离所述细胞。

49.权利要求46的方法，其中所述受试者患有肿瘤。

50.权利要求49的方法，其中所述肿瘤是多发性骨髓瘤、白血病或淋巴瘤。

51.权利要求46的方法，其中所述受试者是人类。

52.权利要求46的方法，进一步包括在将所述细胞施用于所述受试者之前：

使所述细胞与抗CD3/抗CD28珠粒接触；

在低氧条件下孵育所述细胞；和

在常氧条件下孵育所述细胞。

53.权利要求52的方法，其中所述细胞在低氧条件下孵育大约0.5至大约4天。

54.权利要求52的方法，其中所述细胞在常氧条件下孵育大约0.5至大约4天。

免疫检查点嵌合受体疗法

[0001] 相关申请的交叉参考

本申请要求2015年6月29日提交的美国临时申请号62/186,108的优先权,其经此引用全文并入本文。

[0002] 发明背景

绝大多数恶性肿瘤患者将死于其疾病。治疗这些患者的一种方法是通过嵌合抗原受体(CAR)表达来基因修饰T细胞以靶向在肿瘤细胞上表达的抗原。CAR是设计为以不依赖于人白细胞抗原的方式识别细胞表面抗原的抗原受体。在采用CD19靶向方法取得的成功之外,使用表达CAR的基因修饰细胞治疗其它恶性肿瘤的尝试仅取得了有限的成功。

[0003] 近来,靶向CTLA-4(伊匹单抗(ipilimumab))和PD-1(纳武单抗(nivolumab),、派姆单抗(pembrolizumab))的检查点抑制抗体在治疗包括转移性黑素瘤、非小细胞肺癌(NSCLC)和霍奇金氏淋巴瘤的各种恶性肿瘤中显示出相当大的活性。这些数据证明了检查点阻断如何通过克服T细胞无反应性而成为有效免疫疗法的主要障碍。

[0004] 发明概述

在一些方面,该实施方案涉及嵌合跨膜蛋白,其包含抑制性受体的胞外域和可以激活免疫反应的胞内信号传导域。该胞外域可以是例如来自CTLA-4、PD-1、LAG-3或Tim-3的胞外域。该胞内信号传导域可以是例如CD3ζ、4-1BB或CD28的胞内信号传导域。在一些方面,该实施方案涉及编码如本文中所述的嵌合跨膜蛋白的核酸。

[0005] 在一些方面,该实施方案涉及细胞,其包含编码如本文中所述的嵌合跨膜蛋白的核酸。在一些方面,该实施方案涉及细胞,其包含如本文中所述的嵌合跨膜蛋白。

[0006] 在一些方面,该实施方案涉及制造重组细胞的方法,其包括用编码如本文中所述的嵌合跨膜蛋白的核酸转染细胞。

[0007] 在一些方面,该实施方案涉及在受试者中提高免疫反应的方法,其包括向受试者施用如本文中所述的重组细胞。在一些方面,该实施方案涉及在受试者中治疗肿瘤的方法,其包括向受试者施用如本文中所述的重组细胞。

[0008] 附图概述

图1显示了编码嵌合跨膜蛋白的核苷酸序列(SEQ ID NO:1),所述嵌合跨膜蛋白包含来自CD8的前导肽(“CD8a LP”)、小鼠PD-1的胞外域(“PD-1ECD”)和小鼠4-1BB的跨膜与胞内域(分别为“4-1BB TM”和“4-1BB ICD”)。还显示了该核苷酸序列的反向互补(SEQ ID NO:2)。优化密码子以用于在小鼠淋巴细胞中表达。

[0009] 图2显示了编码嵌合跨膜蛋白的核苷酸序列(SEQ ID NO:3),所述嵌合跨膜蛋白包含来自CD8的前导肽(“CD8a LP”)、人PD-1的胞外域(“PD-1ECD”)和人4-1BB的跨膜与胞内域(分别为“4-1BB TM”和“4-1BB ICD”)。还显示了该核苷酸序列的反向互补(SEQ ID NO:4)。优化密码子以用于在人淋巴细胞中表达。

[0010] 图3显示了Lenti-X 293T细胞的流式细胞仪结果,使用下面的实施例2中描述的转染方案,用mCherry基因和编码包含PD-1的胞外域的嵌合跨膜蛋白的核酸(SEQ ID NO:1)转染所述细胞。图3表明,该核酸在293T细胞中表达。

[0011] 图4显示了Lenti-X 293T细胞的流式细胞仪结果，使用下面的实施例1中描述的转导方案，用mCherry基因和编码包含PD-1的胞外域和4-1BB的胞内结构域的嵌合跨膜蛋白的核酸(SEQ ID NO:1)转导所述细胞。细胞在6孔板的1个孔(含有1.9毫升病毒)中转导。图4表明，该核酸在293T细胞中表达。

[0012] 图5显示了Lenti-X 293T细胞的流式细胞仪结果，使用下面的实施例1中描述的转导方案，用mCherry基因和编码包含PD-1的胞外域和4-1BB的胞内结构域的嵌合跨膜蛋白的核酸(SEQ ID NO:1)转导所述细胞。细胞在6孔板的1个孔(0.38毫升病毒)中转导。图5表明，该核酸在293T细胞中表达。

[0013] 图6，A栏和B栏显示了包含具有PD-1胞外域、4-1BB跨膜结构域和4-1BB胞内结构域的嵌合受体的MIL不会负面地影响肿瘤特异性。

[0014] 发明详述

CAR疗法迄今已显示出显著的希望。靶向慢性淋巴细胞性白血病(CLL)和近来靶向急性淋巴细胞性白血病(ALL)的CD19CAR已经取得了显著的成功。有趣的是，靶向其它抗原的CAR还未提供类似的临床反应。此类抗原靶向方法的一个局限在于其仅限于表达特定表面受体的疾病的治疗适用性，以及导致具有抗原损失变体的复发的靶向单一肿瘤抗原的限制。

[0015] 肿瘤免疫学中的一个主要障碍是诱导肿瘤特异性耐受，其限制了许多基于细胞的方法的固有抗肿瘤功效。最近的研究通过靶向导致批准用于转移性黑素瘤的抗-CTLA-4和抗-PD-1的检查点抑制剂显示了明显的临床功效。在一些方面，该实施方案涉及嵌合受体，包含表达检查点抑制剂的胞外域和激活的胞内结构域。其具有将致耐受机制劫持到激活信号中的优点。这种方法可用于其中T细胞无反应性是疾病发病机理的主要方面且其中由内源性T细胞受体库提供抗原特异性的所有临床情况。

[0016] 在一些方面，该实施方案涉及嵌合跨膜蛋白，包含抑制性受体的胞外域、跨膜结构域和胞内信号传导域。在一些实施方案中，该胞内信号传导域可以激活免疫反应。该胞内信号传导域可以包含胞内信号蛋白的一部分。在一些实施方案中，该胞内结构域可以用于维持细胞(如T细胞的)的活化。

[0017] 在一些实施方案中，该胞外域可以将信号转导到胞内信号传导域中。例如，该胞外域可以在结合天然抑制性受体的激动剂时将信号转导至胞内信号传导域。

[0018] 信号转导可以包含蛋白的低聚。低聚可以包括同源低聚或异源低聚。低聚可以包括蛋白的二聚，即与第二嵌合跨膜蛋白的同源二聚或与不同蛋白的异源二聚。

[0019] 信号转导可以包含磷酸化。例如，该胞内信号传导域可以包含激酶活性和/或磷酸化位点。信号转导可以包含自磷酸化，例如该胞内信号传导域的自磷酸化。

[0020] 在一些实施方案中，该蛋白包含跨膜结构域。在一些实施方案中，该蛋白是整合膜蛋白。例如，该蛋白可以是1型膜蛋白、2型膜蛋白或多重跨膜蛋白。在一些实施方案中，该蛋白包含该抑制性受体的跨膜结构域。在一些实施方案中，该蛋白包含该胞内信号蛋白的跨膜结构域。该嵌合跨膜蛋白可以包含信号肽，例如使胞外域跨越细胞膜移位。在一些实施方案中，该跨膜结构域包含IISFFLALTSTALLFLLFFLTLRFSVV的序列(SEQ ID NO:5)。在一些实施方案中，该嵌合跨膜蛋白包含源自CD8的信号肽。在一些实施方案中，该信号肽包含CD8前导肽。在一些实施方案中，该信号肽包含MALPVTALLLPLALLLHAARP(SEQ ID NO:6)。

[0021] 在一些实施方案中，该胞外域是抑制性受体的胞外域。在一些实施方案中，该胞外

域包含配体结合域，例如该抑制性受体的激动剂结合域。在一些实施方案中，该胞外域包含足够的结构以在响应配体结合时跨膜转导信号。不受任何特定理论的束缚，对于通过多价配体介导的低聚来转导信号的抑制性受体而言，仅配体结合域的存在可能是足以在响应配体结合时跨膜转导信号的结构。不受任何特定理论的束缚，对于通过改变相对于细胞膜的跨膜结构域取向转导信号的抑制性受体而言，该胞外域可能需要在配体结合域与跨膜结构域之间的天然结构以在响应配体结合时跨膜转导信号。例如，胞外域可以包含由其配体结合域至其跨膜结构域的抑制性受体的天然序列。

[0022] 该天然抑制性受体可以是人抑制性受体或小鼠抑制性受体。因此，该胞外域可以包含人或小鼠氨基酸序列。在一些实施方案中，选择该天然抑制性受体的来源以匹配受治疗的受试者的物种，例如以避免针对该嵌合跨膜蛋白的免疫反应。尽管如此，例如为了方便起见，该天然抑制性受体可以选自不同的物种。因此，相对于该蛋白在其中表达的细胞的物种或向其施用该蛋白的受试者的物种，该嵌合蛋白可以是或不是异种衍生的。

[0023] 在一些实施方案中，该天然抑制性受体选自在结合天然激动剂时降低免疫活性的蛋白。例如，该天然抑制性受体可以在结合天然激动剂时降低T细胞增殖、T细胞存活、细胞因子分泌或免疫溶细胞活性。该天然抑制性受体可以是淋巴细胞抑制性受体(即可以在淋巴细胞如T细胞上表达的抑制性受体)。例如，该天然抑制性受体可以在T细胞上表达，并且激动剂与天然抑制性受体的结合可能引起不利于T细胞增殖、T细胞存活、细胞因子分泌或免疫溶细胞活性的细胞信号传导。

[0024] 在一些实施方案中，该天然抑制性受体可以是CTLA-4(细胞毒性T淋巴细胞相关蛋白4;CD152)、PD-1(程序性细胞死亡蛋白1;CD279)、LAG-3(淋巴细胞激活基因3;CD223)或Tim-3(T细胞免疫球蛋白粘蛋白-3)。因此，在一些实施方案中，该胞外域可以是来自CTLA-4、PD-1、LAG-3或Tim-3的胞外域。该抑制性受体可以是PD-1。在一些实施方案中，该跨膜蛋白包含PD-1的胞外域。在一些实施方案中，该胞外域的序列包含

PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQT

DKLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPK。

AQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLV.(SEQ ID NO:7)

[0025] 在一些实施方案中，该胞内信号传导域是胞内信号蛋白的信号传导域。在一些实施方案中，该胞内信号传导域可以包含激酶活性或磷酸化位点。在一些实施方案中，该胞内信号传导域可以激活信号分子，如激酶或磷酸化酶，例如在跨越细胞膜的信号转导之后。该胞内信号传导域可以通过下游激酶或磷酸化酶发出信号。

[0026] 该胞内信号蛋白可以是人蛋白或小鼠蛋白。因此，该胞内信号传导域可以包含人或小鼠氨基酸序列。在一些实施方案中，选择该胞内信号蛋白以匹配受试者和用于治疗的细胞的物种，例如使得该信号传导域可以利用该细胞的胞质系统以激活下游的信号分子。尽管如此，如上所述，例如为了方便起见，该胞内信号蛋白可以选自不同的物种。

[0027] 在一些实施方案中，该胞内信号蛋白提高免疫活性。因此，经由嵌合跨膜蛋白的信号转导可以导致提高免疫活性的信号级联，其中该胞内信号传导域介导胞内信号级联反应。在一些实施方案中，该胞内信号蛋白可以增强T细胞增殖、T细胞存活、细胞因子分泌或免疫溶细胞活性。在一些实施方案中，该胞内信号蛋白是跨膜蛋白，或者该胞内信号蛋白可以结合天然跨膜蛋白。该胞内信号蛋白可以是淋巴细胞蛋白(即该胞内信号蛋白可以在淋

巴细胞，如T细胞上表达)。

[0028] 在一些实施方案中，该胞内信号蛋白是CD3ζ(T细胞表面糖蛋白CD3ζ链;CD247)、4-1BB(肿瘤坏死因子受体超家族成员9;CD137)或CD28(T细胞特异性表面糖蛋白CD28;Tp44)。因此，该胞内信号蛋白可以包含来自CD3ζ、4-1BB或CD28的信号传导域。该胞内信号蛋白可以是4-1BB。因此，该胞内信号蛋白可以包含来自4-1BB的信号传导域。在一些实施方案中，该胞内结构域包含

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL(SEQ ID NO:8)。

[0029] 在一些实施方案中，该嵌合跨膜蛋白包含自杀结构域，即，以杀死包含该蛋白的重组细胞。该自杀结构域可以包含胸苷激酶活性或半胱天冬酶活性。例如，该自杀结构域可以是胸苷激酶或半胱天冬酶。在一些实施方案中，该自杀结构域是HSV胸苷激酶(“HSV-TK”)的胸苷激酶结构域，或者该自杀结构域包含半胱天冬酶9的一部分。

[0030] 在一些方面，该实施方案涉及编码如本文中所述的嵌合跨膜蛋白的核酸分子。该核酸分子可以包含启动子，其中该启动子可操作地连接到编码该嵌合跨膜蛋白的核苷酸序列上，例如用于在重组细胞中表达嵌合跨膜蛋白。在一些实施方案中，该启动子是组成型启动子。在一些实施方案中，该启动子是细胞特异性启动子。在一些实施方案中，该启动子是组织特异性启动子。

[0031] 该核酸分子可以包含SEQ ID NO:1、SEQ ID NO:2、SEQ ID NO:3或SEQ ID NO:4中所示的序列。该核酸分子在SEQ ID NO:1、SEQ ID NO:2、SEQ ID NO:3或SEQ ID NO:4中所示的序列中可以包含至少大约100、200、300、400、500、600或700个连续的核苷酸。该核酸分子可以包含与SEQ ID NO:1、SEQ ID NO:2、SEQ ID NO:3或SEQ ID NO:4中所示的核苷酸序列具有至少大约90％、91％、92％、93％、94％、95％、96％、97％、98％或99％的序列同源性的核苷酸序列。该核酸分子可以包含与SEQ ID NO:1、SEQ ID NO:2、SEQ ID NO:3或SEQ ID NO:4中所示的核苷酸序列中至少大约100、200、300、400、500、600或700个连续的核苷酸具有至少大约90％、91％、92％、93％、94％、95％、96％、97％、98％或99％的序列同源性的核苷酸序列。例如，该核酸分子可以包含与SEQ ID NO:3中所示的核苷酸序列中至少100个连续的核苷酸具有至少95％的序列同源性的核苷酸序列。

[0032] 在一些实施方案中，该核酸分子编码如本文中和/或附图中所述的氨基酸序列。在一些实施方案中，该核酸分子编码包含SEQ ID NO:5、SEQ ID NO:6、SEQ ID NO:7、SEQ ID NO:8、SEQ ID NO:9、SEQ ID NO:10或SEQ ID NO:11中所示一种或多种氨基酸序列的氨基酸序列。在一些实施方案中，该核酸分子可以包含与本文中和/或附图中所述的核苷酸序列具有至少大约90％、91％、92％、93％、94％、95％、96％、97％、98％或99％的序列同源性的编码氨基酸序列的核苷酸序列。同源性在蛋白质的情况下可以是同一性或类似性。序列同源性在核酸分子的情况下指的是序列同一性。同源性可以通过利用常规工具如Expasy、BLASTp、Clustal等等采用默认设置来使用。

[0033] 在一些实施方案中，该嵌合跨膜蛋白包含下表中所示的一个或多个氨基酸序列：

序列	SEQ ID NO
IISFFLALTSTALLFLLFFLTLRFSVV	5
MALPVTALLLPLALLLHAARP	6
PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTC SFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQ PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTY LCGAISLAPKAQIKESLRAELRVTERRAEVPTAHP SPSPRPAGQFQTLV	7
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPE EEEGGCEL	8
IISFFLALTSTALLFLLFFLTLRFSVVKRGRKKLLY IFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	9
PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTC SFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQ PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTY LCGAISLAPKAQIKESLRAELRVTERRAEVPTAHP SPSPRPAGQFQTLVIISFFLALTSTALLFLLFFLTLR FSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSC RFPEEEEGGCEL	10
MALPVTALLLPLALLLHAARPPGWFLDSPDRPW NPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNW YRMSPSNQTDKLAAFPEDRSQPGQDCRFRVTQLP NGRDFHMSVVRARRNDSGTYLCGAISLAPKAQI KESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTL VIISFFLALTSTALLFLLFFLTLRFSVVKRGRKKLL YIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	11

在一些实施方案中，该嵌合跨膜蛋白包含与本文中所示氨基酸序列之一具有至少大约90％、91％、92％、93％、94％、95％、96％、97％、98％或99％的序列同源性的氨基酸序列。

[0034] 在各种实施方案中可以包括本文中所述的氨基酸序列的变体。术语“变体”是指其中与蛋白或多肽的氨基酸序列相比存在一个或多个(例如1个、2个、3个、4个等等)氨基酸置换、缺失和/或插入的蛋白或多肽，并且该术语包括蛋白或多肽的天然存在的等位基因变体和可变剪接变体。术语“变体”包括用类似或同源的氨基酸或不同的氨基酸取代氨基酸序列中的一个或多个氨基酸。一些变体包括在氨基酸序列中一个或多个氨基酸位置处的丙氨酸置换。其它置换包括对蛋白的整体净电荷、极性或疏水性几乎没有影响或没有影响的保守性置换。保守性置换可能对嵌合跨膜蛋白的功能具有微小的影响。在一些实施方案中，如实施例3中所述，该功能可以是在淋巴细胞，例如骨髓浸润性淋巴细胞(MIL)中表达时蛋白的特异性。本领域技术人员通过使用与实施例3相同或类似的方案与本文中提供的序列进行比较来确定置换是否影响嵌合跨膜蛋白的功能。非限制性的示例性保守性置换显示在下表中。根据一些实施方案，嵌合跨膜蛋白与本文中描述的氨基酸序列具有至少90％、91％、92％、93％、94％、95％、96％、97％、98％或99％的序列同一性。

[0035] 保守性氨基酸置换

碱性：	精氨酸 赖氨酸 组氨酸
酸性：	谷氨酸 天冬氨酸
非荷电极性：	谷氨酰胺 天冬酰胺 丝氨酸 苏氨酸 酪氨酸
非极性：	苯丙氨酸 色氨酸 半胱氨酸 甘氨酸 丙氨酸 缬氨酸 脯氨酸 蛋氨酸 亮氨酸 异亮氨酸

下表列出了保守性氨基酸置换的另一种方案。

原始残基	保守性置换
Ala	Gly;Ser;Thr
Arg	Lys;Gln
Asn	Gln;His;Ser
Asp	Glu;Asn
Cys	Ser
Gln	Asn;Ser;Asp;Glu

Glu	Asp;Gln;Lys
Gly	Ala;Pro;Asn
His	Asn;Gln;Tyr
Ile	Leu;Val;Met;Val;Phe
Leu	Ile;Val;Met;Phe
Lys	Arg;Gln
Met	Leu;Tyr;Ile;Val;Phe
Pro	Ser;Thr;Ala;Gly
Phe	Met;Leu;Tyr;Trp
Ser	Thr;Gly;Asn;Asp
Thr	Ser;Asn
Trp	Tyr;Phe
Tyr	Trp;Phe
Val	Ile;Leu;Met;Phe

[0036] 因此,在一些实施方案中,本文中公开的氨基酸序列的1、2、3、4、5、6、7、8、9或10个氨基酸残基用保守性置换进行修饰。在一些实施方案中,仅用保守性置换置换1、2、3、4或5个氨基酸残基。

[0037] 在一些实施方案中,该嵌合跨膜蛋白包含SEQ ID NO:10或SEQ ID NO:11或其变体的序列。SEQ ID NO:10是SEQ ID NO:5、7和8的组合。SEQ ID NO:11是SEQ ID NO:5、6、7和8的组合。在一些实施方案中,用另一信号肽或前导序列置换SEQ ID NO:6的序列,其可以帮助将该嵌合跨膜蛋白转运至胞外膜。在一些实施方案中,该跨膜结构域(例如SEQ ID NO:5)被不同的跨膜蛋白替代。在一些实施方案中,该跨膜结构域是PD-1的跨膜结构域。在一些实施方案中,该跨膜结构域是4-1BB的跨膜结构域。

[0038] 在一些方面,该实施方案涉及包含本文中公开的核酸的重组细胞。在一些实施方案中,该实施方案涉及包含如本文中所述的嵌合跨膜蛋白的重组细胞。在一些实施方案中,该细胞包含嵌合蛋白,所述嵌合蛋白包含SEQ ID NO:5、6、7、8、9、10或11或其变体的蛋白。在一些实施方案中,该细胞是淋巴细胞。该细胞可以是T细胞。在一些实施方案中,该细胞可以是肿瘤浸润的淋巴细胞(“TIL”)或骨髓浸润的淋巴细胞(“MIL”)。

[0039] 在一些实施方案中,与不具有嵌合跨膜蛋白的细胞相比,当将包含本文中描述的嵌合跨膜蛋白的细胞施用于受试者时,其在受试者中持续更长时间和/或保持活性状态更长时间。

[0040] 在一些方面,该实施方案涉及制造重组细胞的方法,包括用本文中所述的核酸分子转染细胞。在一些方面,该实施方案涉及制造重组细胞的方法,包括用编码本文中所述的氨基酸序列的核酸分子转染细胞。该核酸分子可以是质粒。该细胞可以被包含一个或多个本文中所述的核苷酸序列的质粒转染。该细胞还可以被包含该核酸分子的病毒或病毒样颗粒感染。在一些实施方案中,该细胞是TIL或MIL。在一些实施方案中,该MIL是激活的MIL。例如可以通过将它们与抗CD3/抗CD28珠粒和适当的细胞因子一起例如在低氧条件下孵育来激活MIL。在低氧条件下生长MIL的实例例如可以在WO 2016037054中找到,其经此引用全文并入本文。在一些实施方案中,在细胞已经在本文中所述的低氧环境中孵育后,将该核酸分

子转染到该细胞中。在一些实施方案中，在细胞已经在低氧环境中孵育大约1、2、3、4或5天后，将该核酸分子转染到该细胞中。在一些实施方案中，该细胞随后在常氧条件下孵育大约1、2、3、4或5天。

[0041] 在一些实施方案中，根据WO2016037054(经此引用全文并入本文)中描述的方法制备包含该嵌合跨膜蛋白的MIL。在一些实施方案中，该方法可以包括从受试者中移出骨髓中的细胞、淋巴细胞和/或骨髓浸润淋巴细胞(“MIL”)；在低氧环境中孵育该细胞，由此产生激活的MIL；并将激活的MIL施用至该受试者。如本文中所述，可以在抗CD3/抗CD28抗体和细胞因子的存在下激活该细胞。在低氧环境中孵育该MIL之前或之后，可以将编码嵌合跨膜蛋白的核酸分子，例如本文所述的那些之一，转染或感染到细胞中。

[0042] 该低氧环境可以包含少于大约21％的氧，如少于大约20％、19％、18％、17％、16％、15％、14％、13％、12％、11％、10％、9％、8％、7％、6％、5％、4％或少于大约3％的氧。例如，该低氧环境可以包含大约0％的氧至大约20％的氧，如大约0％的氧至大约19％的氧，大约0％的氧至大约18％的氧、大约0％的氧至大约17％的氧、大约0％的氧至大约16％的氧、大约0％的氧至大约15％的氧、大约0％的氧至大约14％的氧、大约0％的氧至大约13％的氧、大约0％的氧至大约12％的氧、大约0％的氧至大约11％的氧、大约0％的氧至大约10％的氧、大约0％的氧至大约9％的氧、大约0％的氧至大约8％的氧、大约0％的氧至大约7％的氧、大约0％的氧至大约6％的氧、大约0％的氧至大约5％的氧、大约0％的氧至大约4％的氧或大约0％的氧至大约3％的氧。在一些实施方案中，该低氧环境包含大约1％至大约7％的氧。在一些实施方案中，该低氧环境为大约1％至大约2％的氧。在一些实施方案中，该低氧环境为大约0.5％至大约1.5％的氧。在一些实施方案中，该低氧环境为大约0.5％至大约2％的氧。该低氧环境可以包含大约20％、19％、18％、17％、16％、15％、14％、13％、12％、11％、10％、9％、8％、7％、6％、5％、4％、3％、2％、1％或大约0％的氧。在一些实施方案中，该低氧环境包含大约7％、6％、5％、4％、3％、2％或1％的氧。

[0043] 在低氧环境中孵育MIL可以包括例如在组织培养基中孵育该MIL至少大约1小时，如至少大约12小时、18小时、24小时、30小时、36小时、42小时、48小时、60小时、3天、4天、5天、6天、7天、8天、9天、10天、11天、12天、13天或甚至至少大约14天。孵育可以包括孵育该MIL大约1小时至大约30天，如大约1天至大约20天、大约1天至大约14天或大约1天至大约12天。在一些实施方案中，在低氧环境中孵育MIL包括在低氧环境中孵育该MIL大约2天至大约5天。该方法可以包括在低氧环境中孵育MIL大约1天、2天、3天、4天、5天、6天、7天、8天、9天、10天、11天、12天、13天或14天。在一些实施方案中，该方法包括在低氧环境中孵育MIL大约3天。在一些实施方案中，该方法包括在低氧环境中孵育MIL大约2天至大约4天。在一些实施方案中，该方法包括在低氧环境中孵育MIL大约3天至大约4天。

[0044] 在一些实施方案中，该方法进一步包括在常氧环境中孵育该MIL，例如在低氧环境中孵育该MIL之后。

[0045] 该常氧环境可以包含至少大约21％的氧。该常氧环境可以包含大约5％的氧至大约30％的氧，如大约10％的氧至大约30％的氧、大约15％的氧至大约25％的氧、大约18％的氧至大约24％的氧、大约19％的氧至大约23％的氧或大约20％的氧至大约22％的氧。在一些实施方案中，该常氧环境包含大约21％的氧。

[0046] 在常氧环境中孵育MIL可以包括例如在组织培养基中孵育该MIL至少大约1小时，

如至少大约12小时、18小时、24小时、30小时、36小时、42小时、48小时、60小时、3天、4天、5天、6天、7天、8天、9天、10天、11天、12天、13天或甚至至少大约14天。孵育可以包括孵育该MIL大约1小时至大约30天，如大约1天至大约20天、大约1天至大约14天、大约1天至大约12天或大约2天至大约12天。

[0047] 在一些实施方案中，在置于常氧环境中之后或在将其置于常氧环境中之前，用编码本文中描述的嵌合跨膜蛋白的核酸分子转染或感染该细胞。

[0048] 在一些实施方案中，通过从受试者提取骨髓样品获得该MIL并如本文中所述培养/孵育该细胞来。在一些实施方案中，将该骨髓样品离心以除去红细胞。在一些实施方案中，不对骨髓样品施以成分分离。在一些实施方案中，该骨髓样品不包含外周血淋巴细胞(“PBL”)，或者该骨髓样品基本不含PBL。选择这些方法用于与被称为TIL的细胞不同的细胞。因此，MIL不是TIL。TIL可以通过本领域技术人员已知的方法来选择，并用本文中描述的核酸分子转染或感染，使得该TIL可以表达本文中描述的嵌合跨膜蛋白。

[0049] 在一些实施方案中，还可以通过用对CD3和CD28的抗体培养来激活该细胞。这可以例如通过用市售或可以由本领域技术人员制得的抗CD3/抗CD28珠粒孵育该细胞来进行。该细胞可以随后铺在板、烧瓶或袋子中。可以通过用95％氮气和5％CO_2气体混合物吹扫低氧室或细胞培养袋3分钟来实现低氧条件。这可以在容器中导致，例如1-2％或更少的O_2气体。随后可以如本文中所述或如WO2016037054(其经此引用并入本文)的实施例中那样培养细胞。

[0050] 在一些实施方案中，提供了包含如本文中所述的嵌合跨膜蛋白的低氧MIL。在一些实施方案中，该低氧MIL在大约0.5％至大约5％的氧气的环境中。在一些实施方案中，该低氧MIL在大约1％至大约2％的氧气的环境中。在一些实施方案中，该低氧MIL在大约1％至大约3％的氧气的环境中。在一些实施方案中，该低氧MIL在大约1％至大约4％的氧气的环境中。低氧MIL是已经在低氧环境(如本文中所述的那些)中孵育一段时间的MIL，如本文中所述的那些。不受任何特定理论的束缚，低氧MIL将经历影响该MIL的抗肿瘤能力的蛋白和/或基因表达的变化。如本文中所述，该低氧MIL还可以在抗CD3/抗CD28珠粒或其它类似的激活试剂的存在下被激活。因此，该低氧MIL也可以是激活的低氧MIL。

[0051] 在一些方面，该实施方案涉及在受试者中提高免疫反应的方法，包括向该受试者施用如本文中所述的重组细胞。在一些实施方案中，该实施方案涉及在受试者中治疗肿瘤的方法，包括向该受试者施用如本文中所述的重组细胞。该肿瘤可以是良性肿瘤、恶性肿瘤或继发性肿瘤。该肿瘤可以是癌症。该肿瘤可以是淋巴瘤或白血病，如慢性淋巴细胞性白血病(“CLL”)或急性淋巴细胞性白血病(“ALL”)。该肿瘤可以是多发性骨髓瘤以及任何实体瘤(例如乳腺癌、前列腺癌、肺癌、食道癌、脑癌、肾癌、膀胱癌、胰腺癌、骨肉瘤等等)。

[0052] 该方法可以包括向受试者施用多个本文中所述的重组细胞。该方法可以包括向受试者施用有效量的本文中所述的重组细胞。

[0053] 在一些实施方案中，该细胞获自该受试者。被转染或感染的细胞可以获自该受试者。该细胞可以如本文中所述获得。例如，施用的细胞对该受试者可以是自体同源的。在一些实施方案中，施用的细胞对该受试者可以是同种异体的。该细胞可以获自该受试者并用编码如本文中所述的嵌合跨膜蛋白的核酸转染或感染。该细胞可以是子细胞，其中子细胞的亲代获自该受试者。该重组细胞可以已经用该核酸转染或感染，或该重组细胞的亲代可

以已经用该核酸转染或感染。在一些实施方案中，转染或感染后的细胞表达包含一种或多种本文中描述的氨基酸序列的蛋白。

[0054] 该方法可以进一步包括制造该重组细胞，其中制造该重组细胞包括用编码嵌合跨膜蛋白的核酸，如本文中所述的那些，来转染或感染细胞。在一些实施方案中，该嵌合跨膜蛋白包含SEQ ID NO:5、6、7、8、9、10或11的任一项所示的氨基酸序列或其变体。类似地，该方法可以进一步包括制造多个重组细胞，其中制造多个重组细胞包括用编码嵌合跨膜蛋白的核酸，如本文中所述的那些，来转染或感染多个细胞。该方法可以进一步包括扩增亲代细胞，例如该重组细胞可以是该亲代细胞的子细胞。该方法可以包括扩增细胞群，例如该方法可以包括向受试者施用多个如本文中所述的重组细胞，所述多个重组细胞的各个细胞可以是亲代细胞的子细胞。

[0055] 该方法可以进一步包括从受试者中分离该细胞或亲代细胞。

[0056] 该方法可以进一步包括分选该细胞，例如通过荧光激活细胞分选(“FACS”)或磁性激活细胞分选(“MACS”)。

[0057] 该细胞可以通过任何合适的途径例如在可药用组合物中施用于受试者。在一些实施方案中，该组合物是无热原的。例如，可以采用本领域已知的任何方法进行细胞的施用。例如，施用可以是胃肠外、静脉内、动脉内、皮下、肌肉内、颅内、眼眶内、眼内、心室内、囊内、脊柱内、脑池内、腹膜内、脑室内或鞘内。对于胃肠外给药，该细胞可以在含有可药用赋形剂或载体的组合物中通过静脉内、皮下或肌肉内注射来施用。该细胞可以配制用于通过注射，例如通过推注或连续输注来进行肠胃外给药。该组合物可以采取诸如在油性或水性赋形剂中的混悬剂、溶液或乳液的形式，并可以含有配制剂，例如悬浮剂、稳定剂和/或分散剂。

[0058] 对于注射给药，可能期望使用在无菌水性赋形剂中的溶液的细胞，所述溶液还可以含有其它溶质，如缓冲剂或防腐剂以及足够量的可药用盐或葡萄糖以使该溶液等渗。在一些实施方案中，该药物组合物可以用可药用载体配制以提供用于注射给药的无菌溶液或混悬剂。特别地，注射剂可以以常规形式以液体溶液或悬浮液形式或以乳液形式来制备。合适的赋形剂是例如水、盐水、右旋糖、甘露糖醇、乳糖、卵磷脂、白蛋白、谷氨酸钠、半胱氨酸盐酸盐等等。此外，如果需要的话，可注射药物组合物可以含有少量的无毒辅助物质，如润湿剂、pH缓冲剂等等。合适的药物载体描述在E.W.Martin的“Remington’s pharmaceutical Sciences”中。

[0059] 该受试者可以是包含免疫细胞的任何生物体。例如，该受试者可以选自啮齿类、犬、猫、猪、绵羊、牛、马和灵长类。该受试者可以是小鼠或人类。

[0060] 该受试者可以患有肿瘤。该肿瘤可以是良性肿瘤、恶性肿瘤或继发性肿瘤。该肿瘤可以是癌症。该肿瘤可以是淋巴瘤或白血病，如慢性淋巴细胞性白血病(“CLL”)或急性淋巴细胞性白血病(“ALL”)。该受试者可以患有胶质母细胞瘤、髓母细胞瘤、乳腺癌、头颈癌、肾癌、卵巢癌、卡波西氏肉瘤、急性骨髓性白血病和B系恶性肿瘤。该受试者可以患有多发性骨髓瘤。

[0061] 在一些实施方案中，该受试者是“需要其”的受试者。本文中所用的短语“需要其”指的是该受试者已被确定或怀疑为需要特定方法或治疗。在一些实施方案中，所述确定可以通过任何诊断手段进行。在本文中描述的任何方法和治疗中，该受试者可以是需要其的受试者。

[0062] 除非上下文另行明确要求，本文中所用的术语如“一个”、“一种”和“该”包括单数和复数指示物。

[0063] 本文中所用的术语“包含”、“具有(have)”、“具有(has)”和“包括”以及它们的结合物在本文中使用时是指“包括但不限于”。虽然就“包含”各种组分或步骤而言(解释为“包括但不限于”)描述了各种组合物和方法，该组合物、方法和装置还可以“基本上由”或“由”各种组分和步骤“组成”，此类术语应解释为限定了基本封闭的成员组。

[0064] 本文中所用的术语“治疗(treat)”、“治疗(treated)”和“治疗(treating)”指的是其中目标是减缓(减少)不期望的生理病症、失调或疾病，或获得有益或期望的临床结果的治疗性处理。对本文中描述的实施方案来说，有益或期望的临床结果包括但不限于缓解症状；减轻病症、失调或疾病的程度；稳定(即不恶化)病症、失调或疾病的状态；延迟病症、失调或疾病的发作或减缓病症、失调或疾病的进展；改善病症、失调或疾病状态或缓解(无论部分还是全部)，无论可检测还是不可检测；改善至少一种可测量的物理参数，不必可以由患者辨别；或增进或改善病症、失调或疾病。由此，“癌症治疗”或“治疗癌症”指的是减轻或改善与癌症或本文中描述的任何其它病症相关的任何原发现象和继发症状的活性。在一些实施方案中，治疗的癌症是本文中所述癌症之一。

实施例

[0065] 下面的实施例说明而非限制本文中描述的方法与组合物。治疗中通常遇到并对本领域技术人员显而易见的各种病症和参数的其它合适的修改与调整在实施方案的精神与范围内。

[0066] 实施例1:CAR转导方案

在转导前16-24小时，将T细胞铺在合适的培养基中并用CD3、CD28和IL-2刺激。该细胞随后放置在孵育箱(37℃/5％CO_2)中过夜。在16-24小时后，在不干扰细胞的情况下尽可能多地除去培养基。随后向细胞中添加CAR病毒并放回孵育箱中放置4-12小时。在4-12小时后，将适当体积的含有IL-2的培养基加回到细胞中并放回到孵育箱中。细胞留在孵育箱中生长(在必要的情况下分开并更换培养基)3-12天。可以通过多种方法来检查CAR转导，包括但不限于流式细胞仪、蛋白质印迹或荧光显微术(如果使用荧光报道基因的话)。

[0067] 实施例2:CAR转染方案

293T细胞在DMEM+10％FBS中每两天传代，传代至少三次，其细胞密度使其不会超过80％汇合。在转染前一天，以24小时后(在转染当天)其为大约80％汇合的密度接种该293T细胞。在转染当天，除去培养基，并加入足够的新鲜培养基以覆盖细胞。在单独的试管中，将VSV-G、Gag、Pol&Rev质粒、转染试剂和CAR质粒混合并在室温下孵育10-20分钟。随后将该混合物逐滴添加到293T细胞中并孵育过夜。在转染后12-24小时，完全更换培养基或添加额外的新鲜培养基。在转染后48小时和72小时，收集来自该细胞的含有病毒的培养基并用新鲜培养基补充该细胞。通过离心或过滤移除收集的培养基中的任何细胞。收集的培养基随后在超速离心机中旋转以使病毒成球。除去过量的培养基，并将病毒重新悬浮在DMEM或HBSS中，分装到无菌试管中并在-80℃下储存直至使用。

[0068] 实施例3:嵌合受体蛋白的存在不会负面地影响MIL功能和生长

如本文中所述激活和扩增获自受试者的MIL。简而言之，在从受试者处获得骨髓样品

后，如WO 2016037054(其经此引用并入本文)中所述，该细胞在低氧条件下在抗CD3/抗CD28珠粒和细胞因子的存在下孵育。随后用包含编码含有SEQ ID NO:11的嵌合跨膜蛋白的核酸分子的病毒感染该MIL。该细胞随后在常氧条件下生长并使其扩增。对照物和感染的MIL与不同的细胞类型接触。无论MIL的扩增还是MIL识别抗原的能力都不受该嵌合跨膜蛋白的存在的负面影响。这些结果表明，向MIL中添加嵌合跨膜蛋白对其功能和生长无害。该结果显示在图6，A栏和B栏中，其来自两名不同的患者。

[0069] 总而言之，本文中提供的实施方案和实施例证明，表达嵌合跨膜蛋白的细胞可以有效地用于治疗癌症和/或调节免疫反应。

[0070] 在本说明书中提及和/或在申请数据表中列出的任何美国专利、美国专利申请公开、美国专利申请、外国专利、外国专利申请和非专利出版物(包括CAS编号)均经此引用以其全文并入本文。

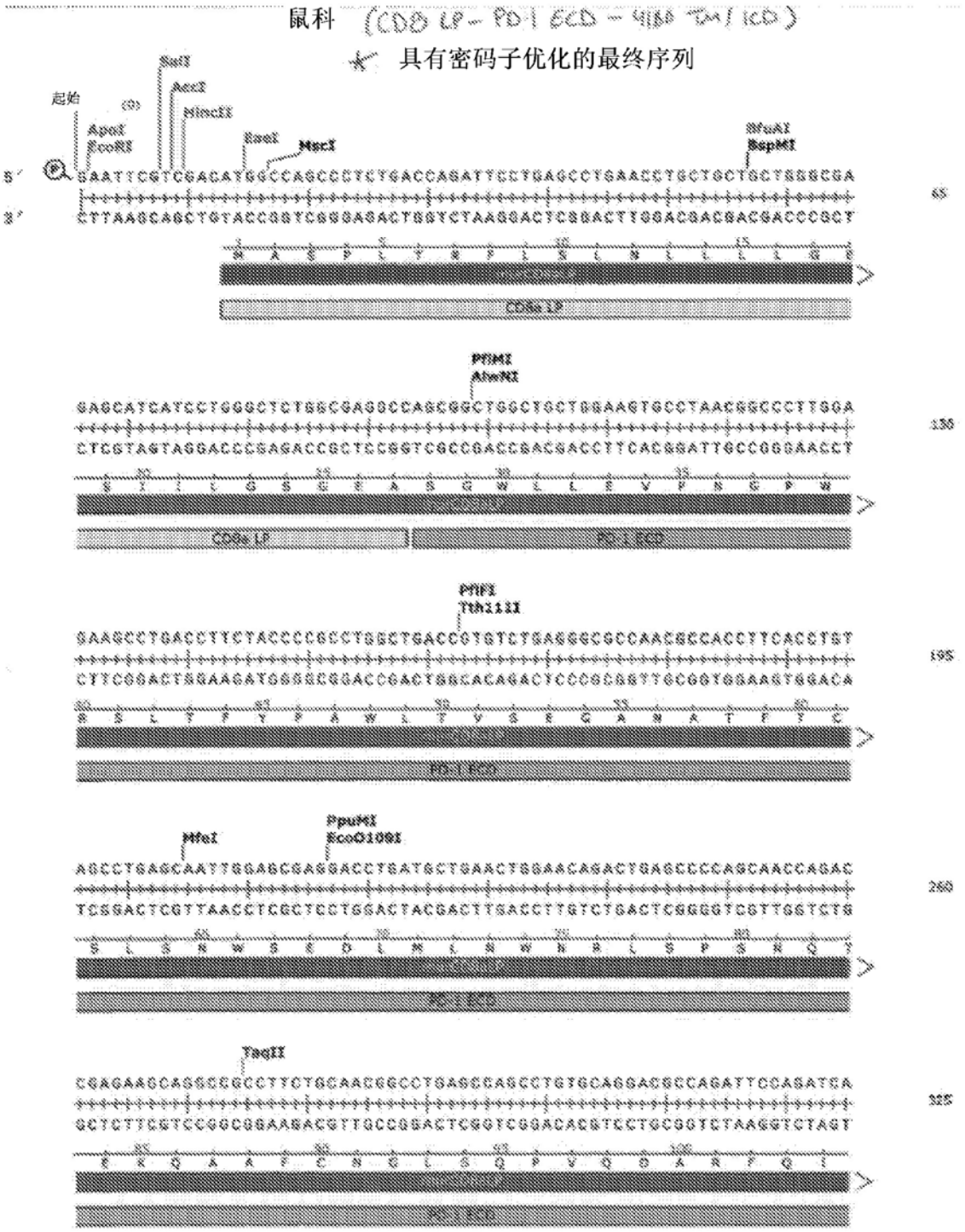

鼠科 (CD8 LP - PD-1 ECD - 4-1BB TM/ICD)
具有密码子优化的最终序列
起始
(D)
ApaI
EcoRI
SacI
AscI
NheI
XbaI
MscI
BfuAI
BspMI
5'
3'
GAATTCGTCGACATGGCCAGCCCTCTGACCAGATTCCTGAGCCTGAACCTGCTGCTGCTGGGCGA
CTTAAGCAGCTGTACCGGTCGGGAGACTGGTCTAAGGACTCGGACTTGGACGACGACGACCCGCT
65
W A S P L T Q I L S L N L L L L G E
CD8a LP
PflMI
AflwNI
GAGCATCATCCTGGGCTCTGGCGAGGCCAGCGGCTGGCTGCTGGAAGTGCCTAACGGCCCTTGGA
CTCGTAGTAGGACCCGAGACCGCTCCGGTCGCCGACCGACGACCTTCACGGATTGCCGGGAACCT
135
S I I L G S G E A S G W L L E V P N G P W
CD8a LP
PD-1 ECD
PflFI
Tth111I
GAAGCCTGACCTTCTACCCCGCCTGGCTGACCGTGTCTGAGGGCGCCAACGCCACCTTCACCTGT
CTTCGGACTGGAAGATGGGGCGGACCGACTGGCACAGACTCCCGCGGTTGCGGTGGAAGTGGACA
195
S S L T F Y P A W L T V S E G A N A T F T C
PD-1 ECD
PflMI
PpuMI
EcoO109I
AGCCTGAGCAATTGGAGCGAGGACCTGATGCTGAACTGGAACAGACTGAGCCCCAGCAACCAGAC
TCGGACTCGTTAACCTCGCTCCTGGACTACGACTTGACCTTGTCTGACTCGGGGTCGTTGGTCTG
260
S L S N W S E D L M L N W N R L S P S N Q T
PD-1 ECD
TaqII
CGAGAAGCAGGCGGCCTTCTGCAACGGCCTGAGCCAGCCTGTGCAGGACGCCAGATTCCAGATGA
GCTCTTCGTCCGGCGGAAGACGTTGCCGGACTCGGTCGGACACGTCCTGCGGTCTAAGGTCTAGT
325
E K Q A A F C N G L S Q P V Q D A R F Q I
PD-1 ECD

图 1

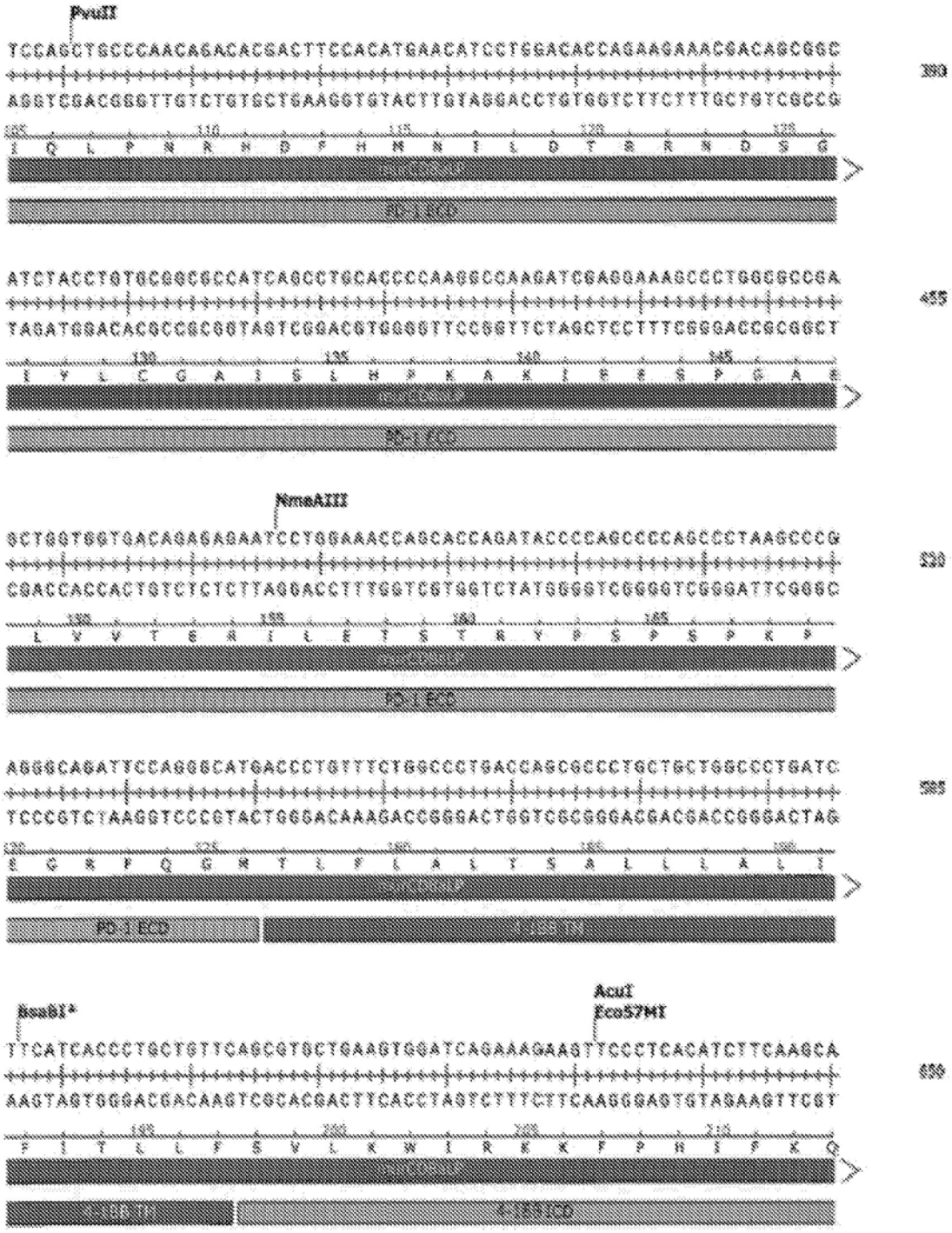

PvuII
TCCAGCTGCCCAACAGACACGACTTCCACATGAACATCCTGGACACCAGAAGAAACGACAGCGGC
AGGTCGACGGGTTGTCTGTGCTGAAGGTGTACTTGTAGGACCTGTGGTCTTCTTTGCTGTCGCCG
I Q L P N R N D F H M N I L D T R R N D S G
PD-1 ECD
390
ATCTACCTGTGCGGCGCCATCAGCCTGCACCCCAAGGCCAAGATCGAGGAAAGCCCTGGCGCCGA
TAGATGGACACGCCGCGGTAGTCGGACGTGGGGTTCCGGTTCTAGCTCCTTTCGGGACCGCGGCT
I Y L C G A I S L H P K A K I E E S P G A E
PD-1 ECD
455
NmeAIII
GCTGGTGGTGACAGAGAGAATCCTGGAAACCAGCACCAGATACCCCAGCCCCAGCCCTAAGCCCG
CGACCACCACTGTCTCTCTTAGGACCTTTGGTCGTGGTCTATGGGGTCGGGGTCGGGATTCGGGC
L V V T E R I L E T S T R Y P S P S P K P
PD-1 ECD
520
AGGGCAGATTCCAGGGCATGACCCTGTTTCTGGCCCTGACCAGCGCCCTGCTGCTGGCCCTGATC
TCCCGTCTAAGGTCCCGTACTGGGACAAAGACCGGGACTGGTCGCGGGACGACGACCGGGACTAG
E G R F Q G M T L F L A L T S A L L L A L I
PD-1 ECD
585
BsaBI*
AcuI
Eco57MI
TTCATCACCCTGCTGTTCAGCGTGCTGAAGTGGATCAGAAAGAAGTTCCCTCACATCTTCAAGCA
AAGTAGTGGGACGACAAGTCGCACGACTTCACCTAGTCTTTCTTCAAGGGAGTGTAGAAGTTCGT
F I T L L F S V L K W I R K K F P H I F K Q
PD-1 ECD
650

图 1(续)

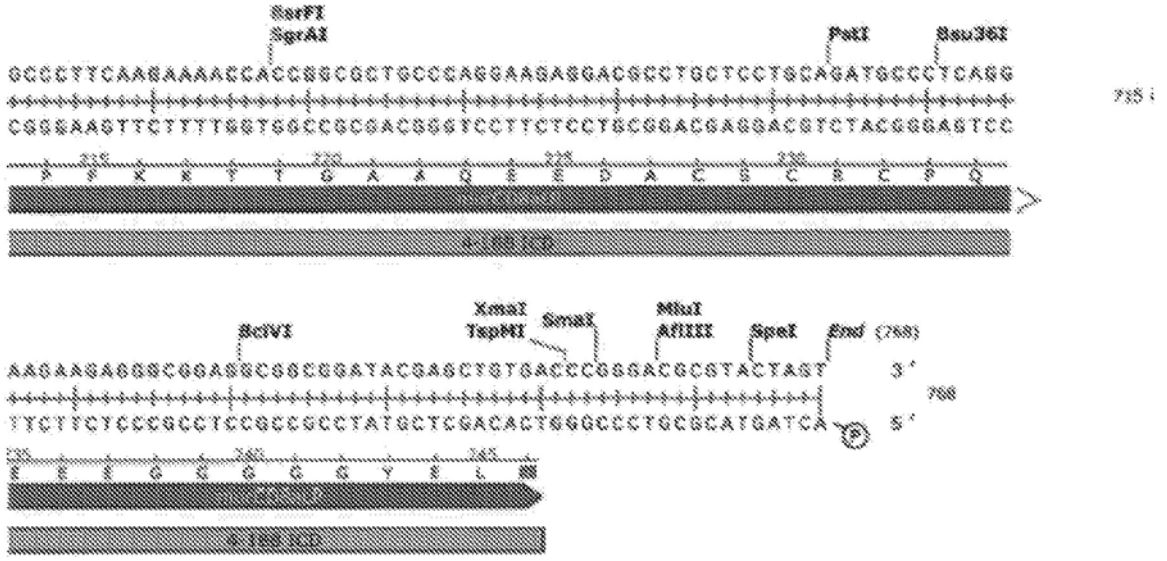

SacII
SgrAI
PstI
Bsu36I
BclVI
XmaI
TspMI
SmaI
MluI
AflIII
SpeI
End
3'
5'

图 1(续)

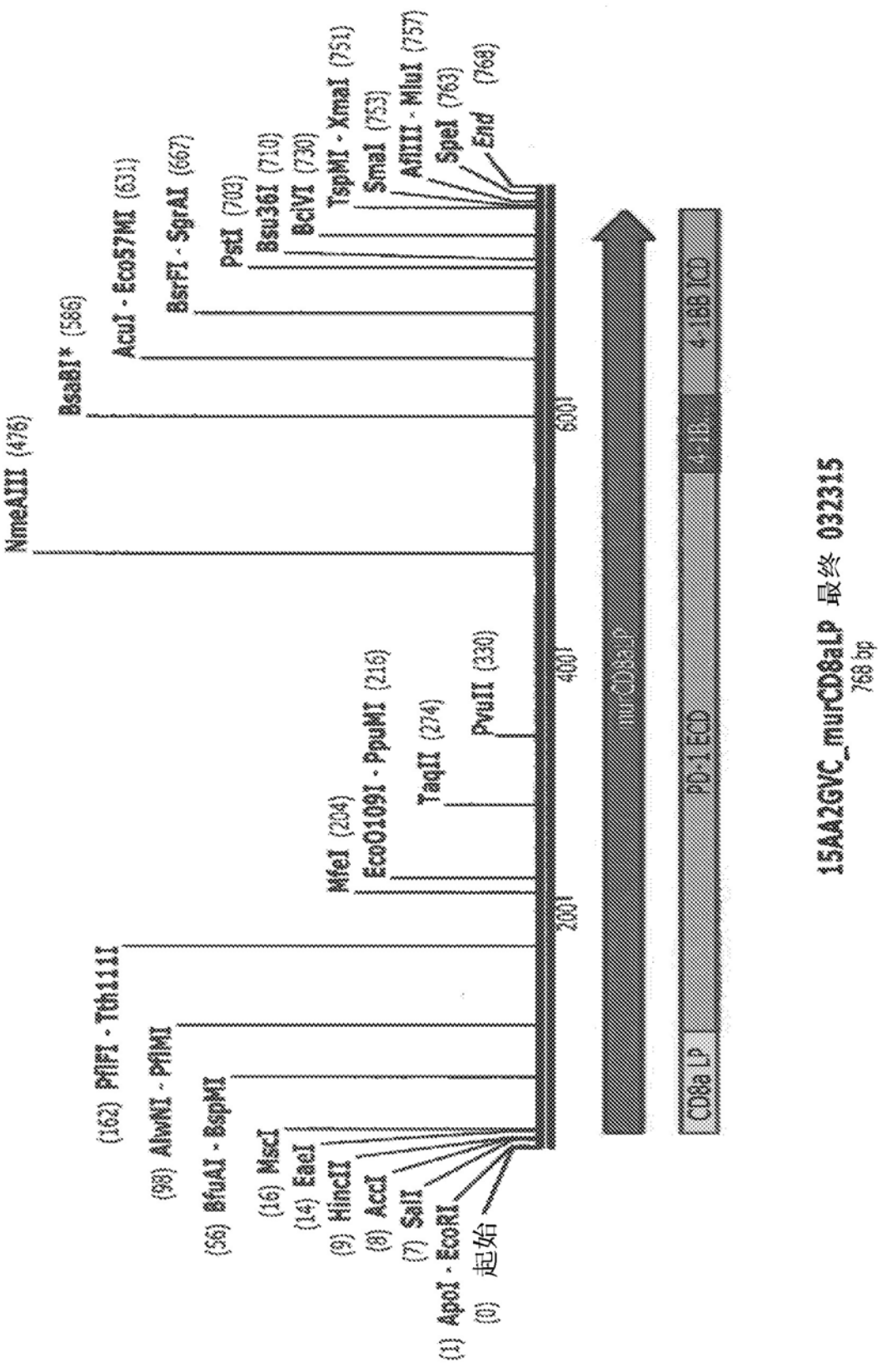

15AA2GVC_murCD8aLP 最终 032315
768 bp
CD8a LP
PD-1 ECD
4-1BB ICD
mu CD3ζ LP
1
200
400
600
(0) 起始
(1) ApoI - EcoRI
(7) SalI
(8) AccI
(9) HincII
(14) EaeI
(16) MscI
(58) BfuAI - BspMI
(98) AlwNI - PflMI
(162) PflFI - Tth111I
MfeI (204)
Eco0109I - PpuMI (216)
TaqII (274)
PvuII (330)
NmeAIII (476)
BsaBI* (586)
AcuI - Eco57MI (631)
BsrFI - SgrAI (667)
PstI (703)
Bsu36I (710)
BclVI (730)
TspMI - XmaI (751)
SmaI (753)
AflIII - MluI (757)
SpeI (763)
End (768)

图 1(续)

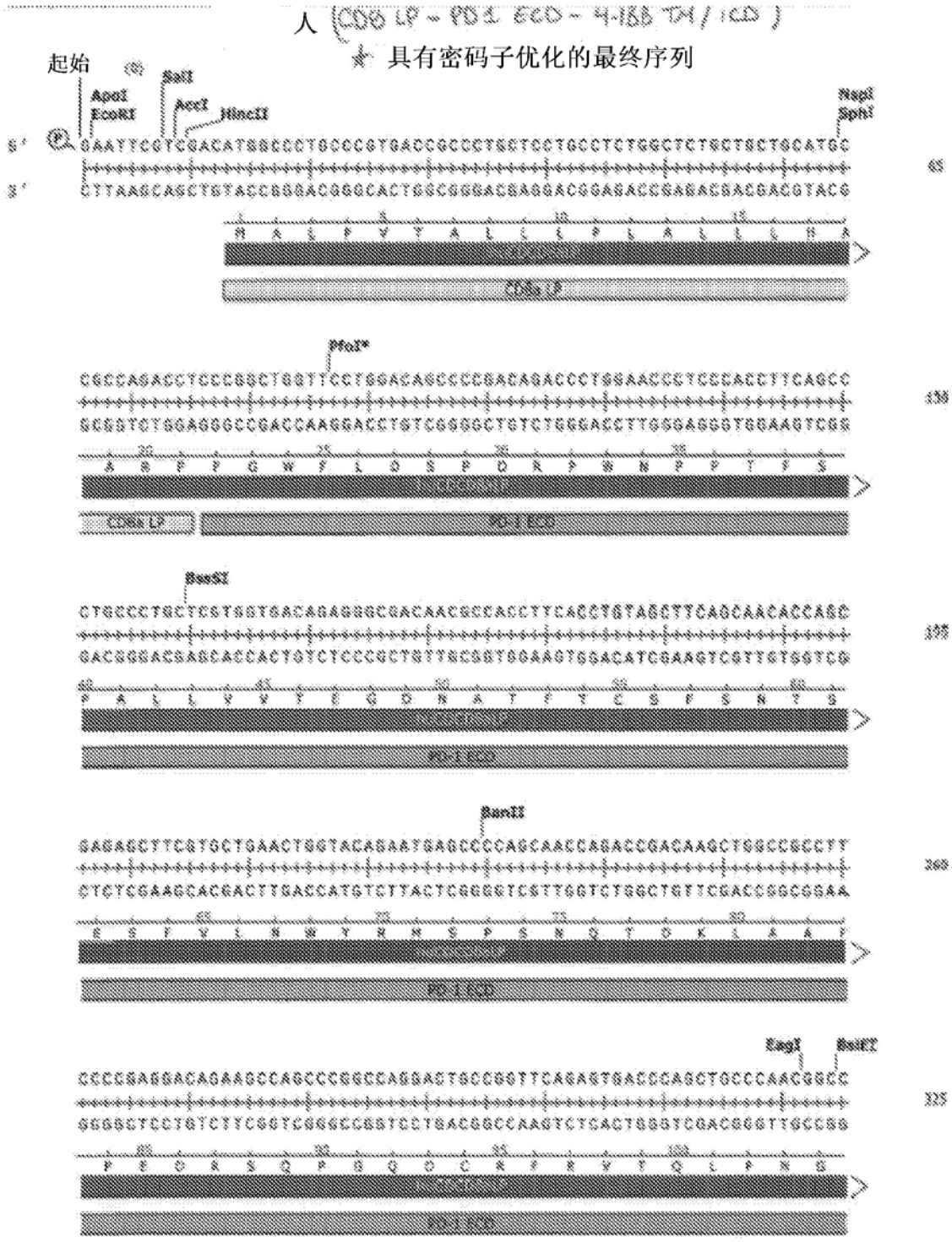

人 (CD8 LP - PD-1 ECD - 4-1BB TM / ICD)
具有密码子优化的最终序列
起始
ApaI
EcoRI
SalI
AvrII
HincII
NspI
SphI
PfoI*
BsmBI
BsmFI
EagI
BsiWI
CD8α LP
PD-1 ECD

图 2

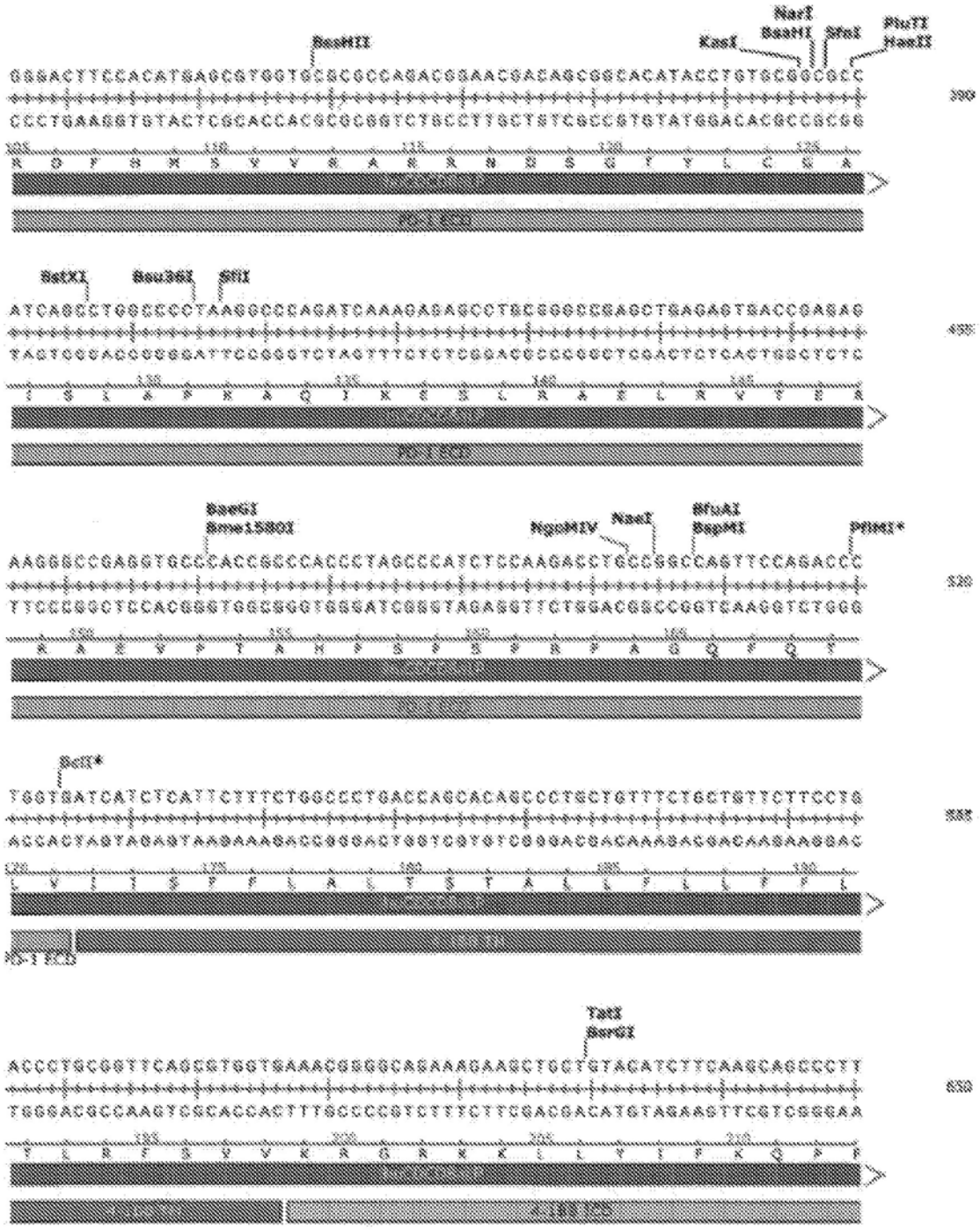

BsaHII
KasI
NarI
BsaHI
SfaI
PluTI
HaeII
390
BstXI
Bau36I
SfiI
450
BaeGI
Bme1580I
NgoMIV
NaeI
BfuAI
BspMI
PflMI*
520
BclI*
580
TaqI
BsrGI
630

图 2(续)

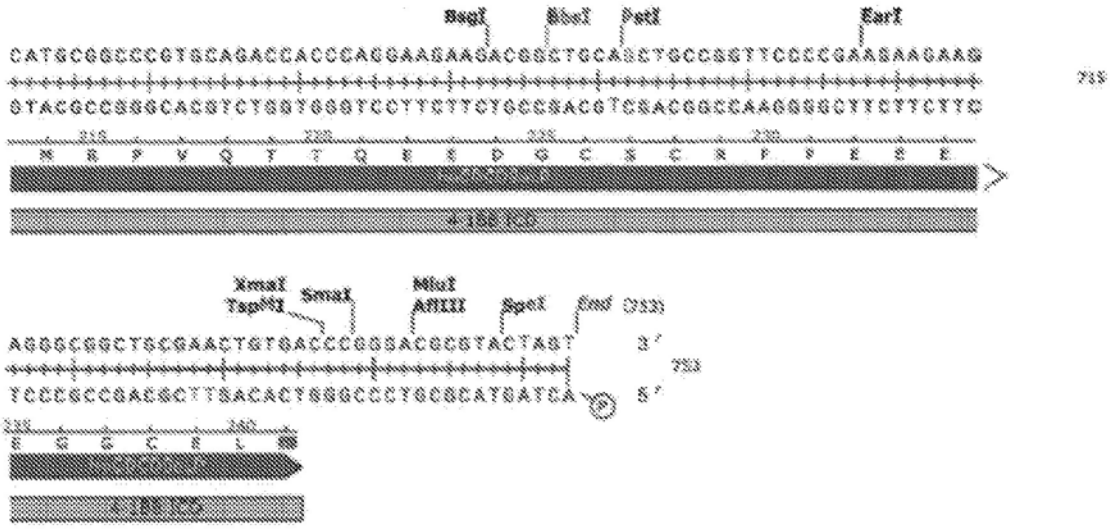

BsaI
BbsI
PstI
EarI
715
XmaI
TaqαI
SmaI
MluI
AflIII
SpeI
EcoRI
753
3′
5′

图 2(续)

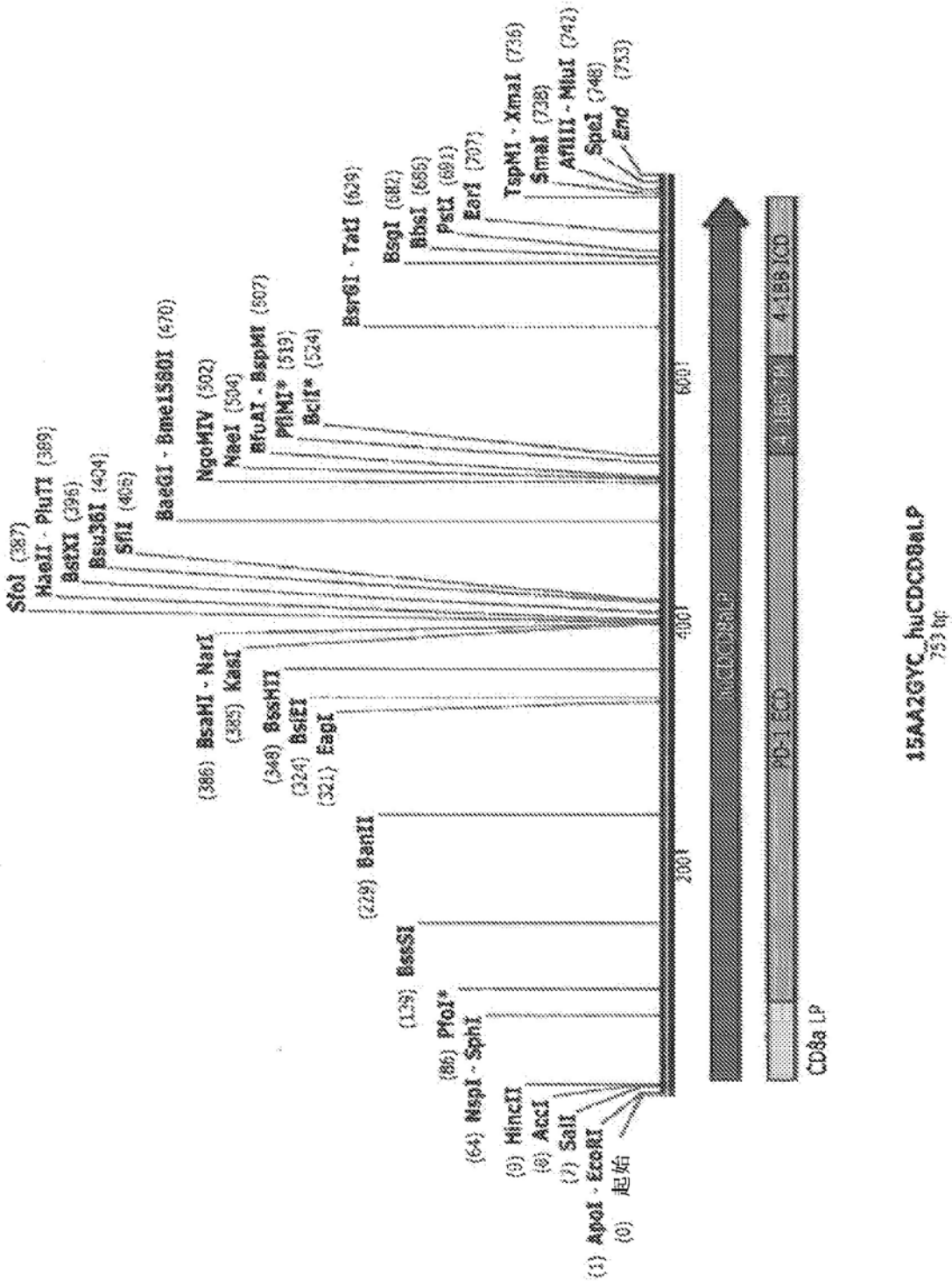

SfoI (387)
HaeII - PluTI (389)
BstXI (395)
Bsu36I (404)
SfiI (405)
BaeGI - BmeI580I (470)
NgoMIV (502)
NaeI (504)
BfuAI - BspMI (607)
PflMI* (519)
BclI* (524)
BsrGI - TatI (629)
BsgI (682)
BbsI (686)
PstI (691)
EarI (707)
TspMI - XmaI (736)
SmaI (738)
AflIII - MluI (742)
SpeI (748)
End (753)
BsaHI - NarI (386)
KasI (385)
BssHII (340)
BstEII (324)
EagI (321)
BanII (229)
BssSI (139)
PfoI* (86)
NspI - SphI (64)
HincII (9)
AccI (8)
SalI (7)
ApaI - EcoRI (1)
起始 (0)
1
200
400
600
CD8α LP
PD-1 ECD
4-1BB TCD
CD3ζ SSD
1EAA2GYC_huCDCD8αLP
753 bp

图 2(续)

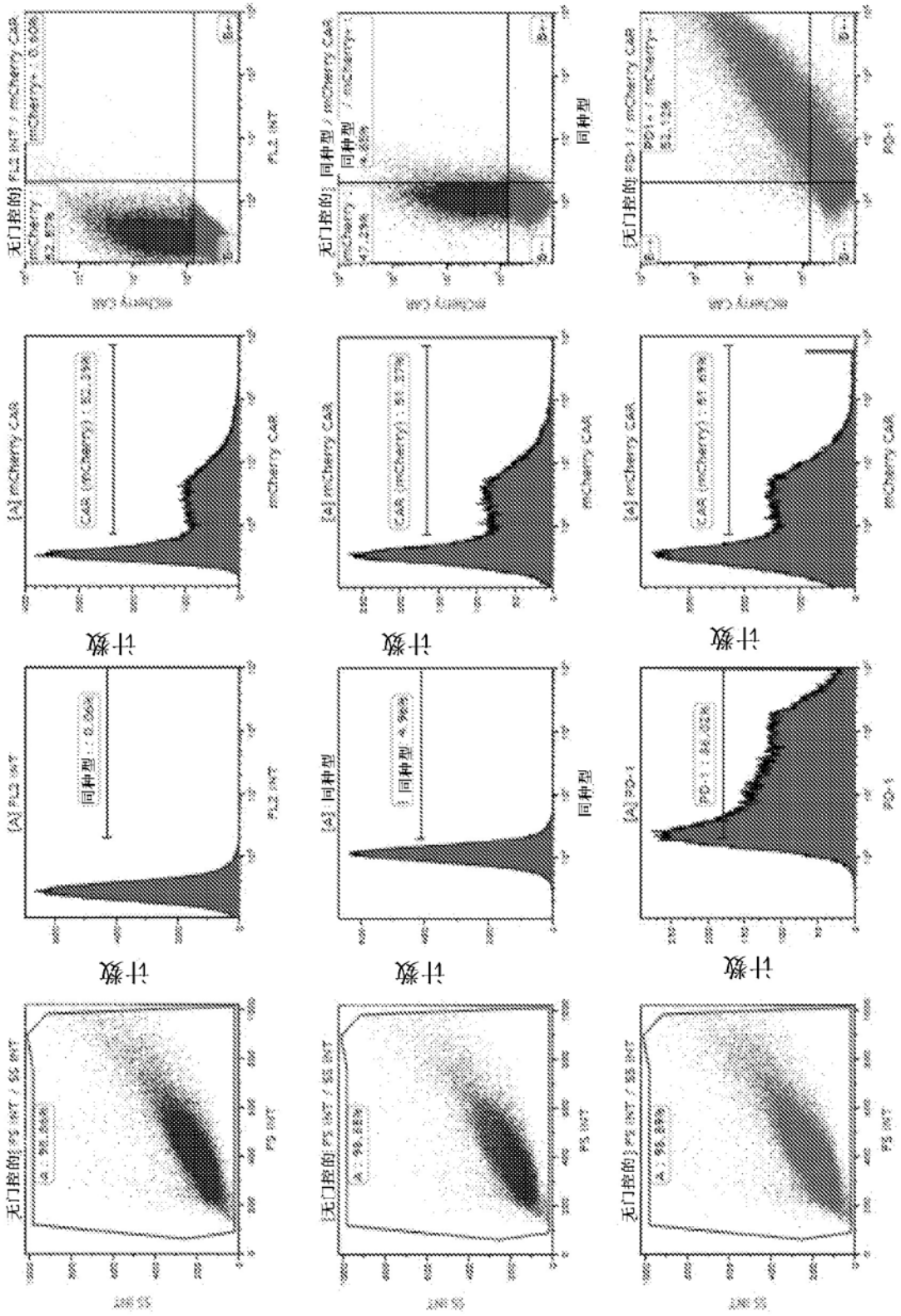


图 3

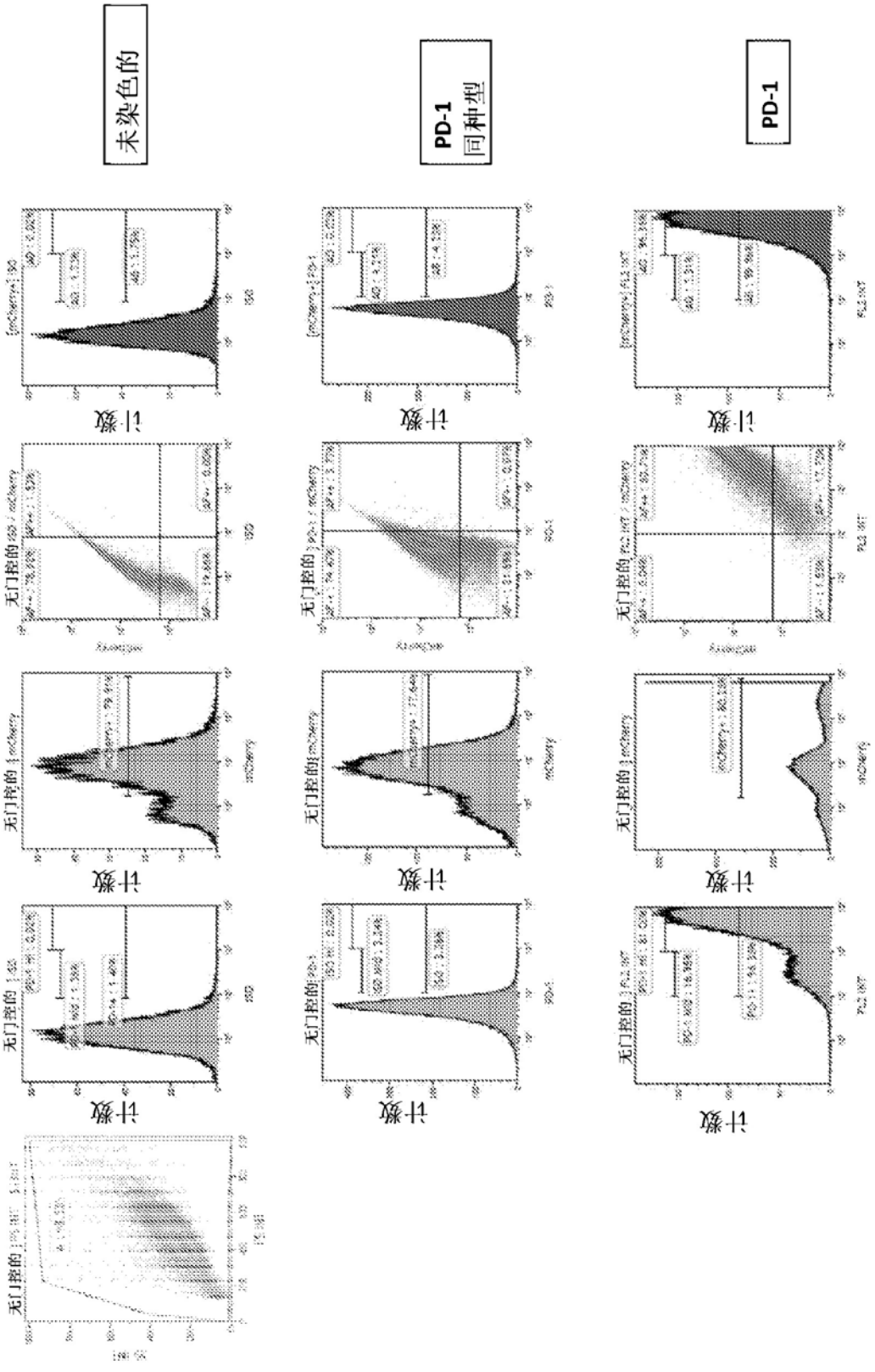


图 4

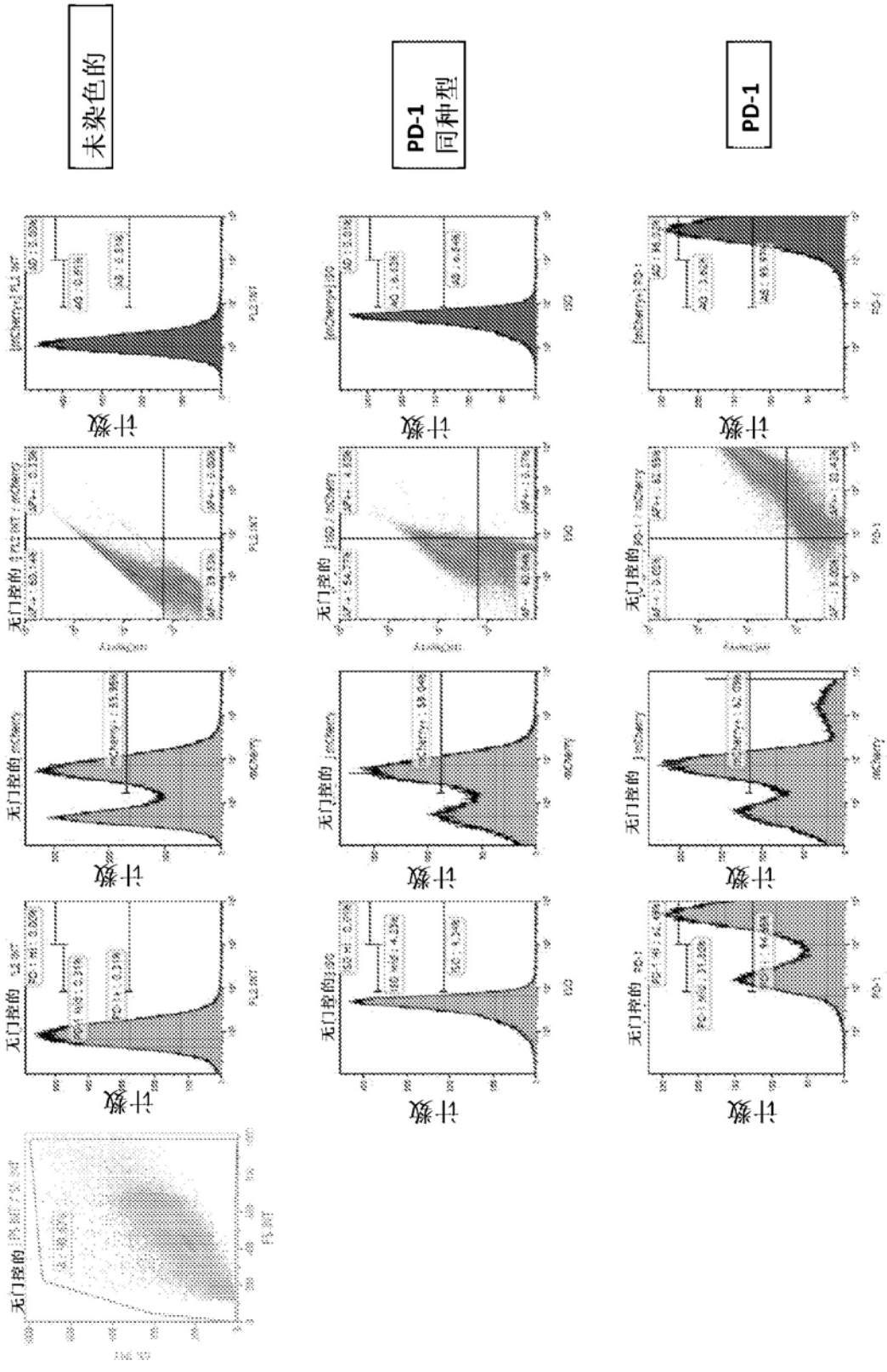
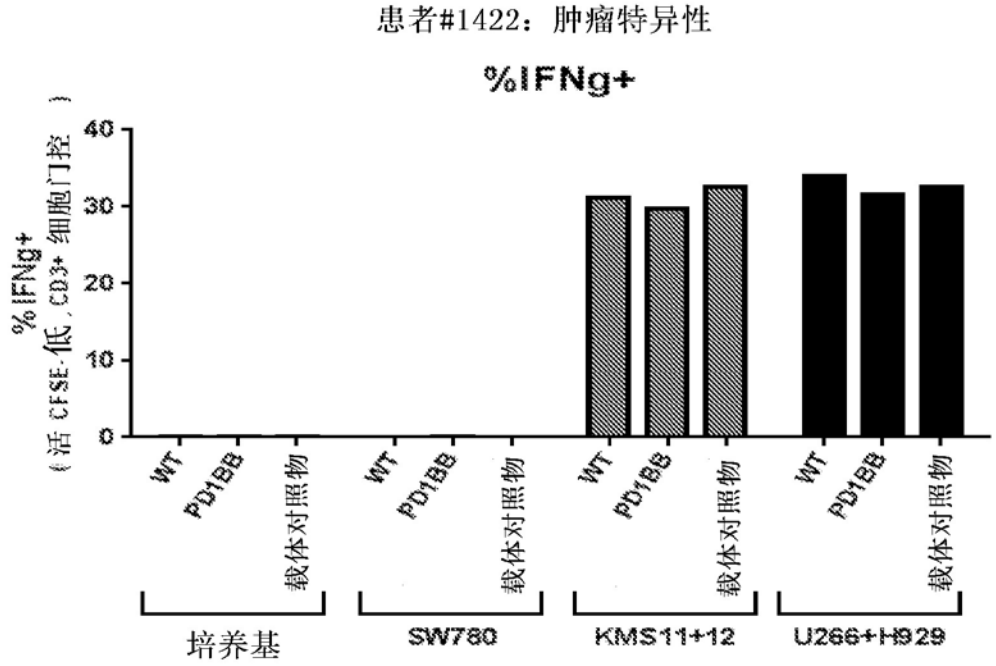


图 5

A.


患者#1422：肿瘤特异性
%IFNg+
%IFNg+
（活 CFSE-低 CD3+ 细胞门控）
40
30
20
10
0
WT
PD1BB
载体对照物
培养基
SW780
KMS11+12
U266+H929

B.

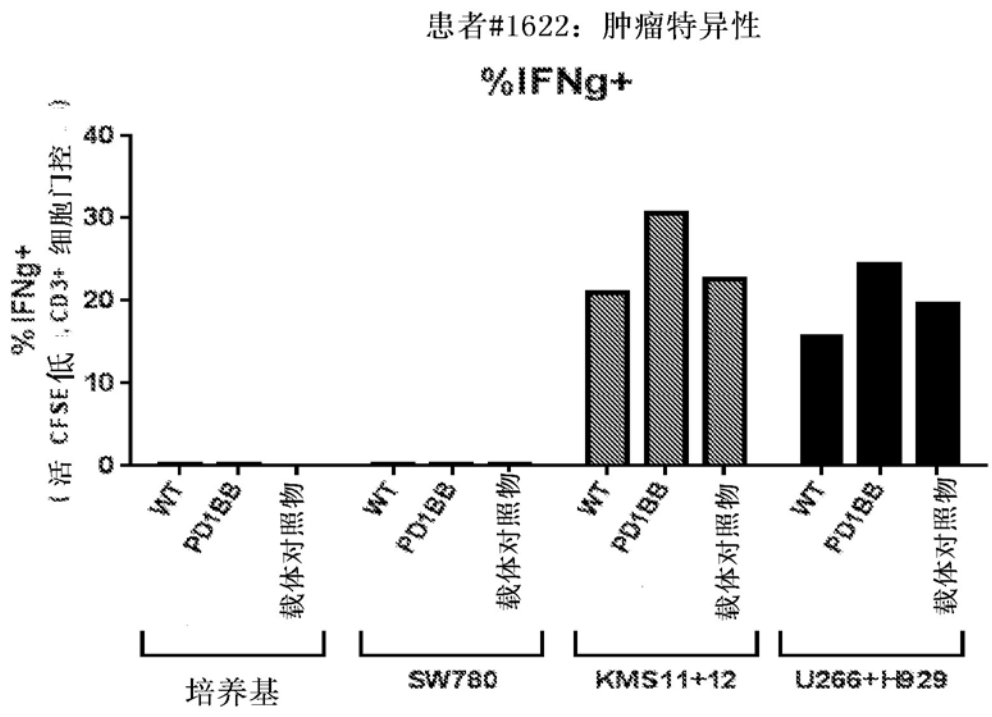

患者#1622：肿瘤特异性
%IFNg+
%IFNg+
（活 CFSE低 CD3+ 细胞门控）
40
30
20
10
0
WT
PD1BB
载体对照物
培养基
SW780
KMS11+12
U266+H929

图 6