

METHOD AND COMPOSITION FOR TREATING DISEASES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from PCT application PCT/CN2023/109440, filed on July 26, 2023, the entire contents of which are hereby incorporated by reference.

TECHNICAL FIELD

The present disclosure relates to a method and composition for preventing and/or treating diseases.

BACKGROUND

A connective tissue disease is any disease that affects the parts of the body that connect the structures of the body together. There are more than 200 different types of connective tissue diseases. They may be inherited, caused by environmental factors, or most often, are of unknown cause. Connective tissue diseases include, but are not limited to Rheumatoid Arthritis (RA), Scleroderma, Granulomatosis with Polyangiitis (GPA), Churg-Strauss Syndrome, Systemic Lupus Erythematosus (SLE), Microscopic Polyangiitis (MPA), Idiopathic inflammatory myopathy (IIM), such as polymyositis (PM), dermatomyositis (DM), amyopathic dermatomyositis (ADM), immune necrotizing myopathy (IMNM), inclusion body myositis (IBM) and juvenile dermatomyositis (JDM), Mixed connective tissue disease (MCTD), undifferentiated connective tissue disease(s). Because there are so many different kinds of connective tissue diseases, symptoms may vary and may affect different parts of the body, and the treatments will vary depending on the person and the disease. Treatments might include vitamin supplements, physical therapy, and medications.

CD19 is a surface protein expressed on B cells, which are immune cells producing an antibody. In patients with connective tissue disease, B cells produce antibodies targeting autologous tissues, resulting in inflammation and tissue impairment.

Autologous, patient-derived chimeric antigen receptor T cell (CAR-T) therapies have shown remarkable efficacy in treating some connective tissue disease (Müller F, *et al*, CD19-targeted CAR T cells in refractory anti-synthetase syndrome. *Lancet*. 2023 Mar 11;401(10379):815-818). By treating with CART targeting CD19, B cells are eliminated selectively, reducing the production of antibodies targeting autologous tissue.

In addition, lymphatic malignancies, including lymphocytic leukemia and lymphoma, are tumors that occur on lymphocytes such as B cells, T cells, and NK cells, and both B cell lymphoma and B lymphocyte leukemia express CD19 antigens. B cell lymphoma comprises Hodgkin's lymphoma(HL) and non-Hodgkin's lymphoma(NHL), and B lymphocyte leukemia comprises acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

CD19-CART cells can recognize CD19 antigens expressed by the B cells, releasing cytokines such as perforin and granzyme, thereby promote to eliminate B cell lymphoma or B lymphocyte leukemia from the organism.

However, autologous CAR-T therapies pose manufacturing challenges and financial burdens due to the need of individualized therapies. Accordingly, attentions have been paid to allogeneic CAR-T therapies, which use allogeneic T cells or “off-the-shelf” T cells and have similar clinical efficacy profiles to autologous products.

While allogeneic CAR-T therapies have the potential to reduce cost of goods and lot-to-lot variability, allogeneic immune cells tend to be subject to recognition and rejection by the host immune system, hence compromise the treatment efficacy of the CAR-T cells. Moreover, allogeneic CAR-T therapies may further suffer from immunosuppression, which can reduce the effector functions and cytotoxicity of the CAR-T cells. Therefore, there is a need for novel adoptive cell therapies with increased effector function and reduced immunogenicity.

SUMMARY

The first aspect of the present disclosure provides a method for preventing and/or treating a condition and/or a disease in a subject in need thereof, comprising administering to the subject

an effective amount of a modified cell and/or a pharmaceutical composition comprising the same;
wherein the condition or the disease is the condition or the disease of a diffuse connective tissue disease, or the condition or the disease of lymphatic malignancies;
wherein the modified cell is, relative to an unmodified counterpart cell:
deficient in an endogenous major histocompatibility complex and an endogenous inhibitory checkpoint molecule;
engineered to express a ligand of the inhibitory checkpoint molecule;
wherein the ligand is a variant of the ligand having increased capability of inducing immunoinhibitory signaling relative to the cognate ligand.

In some embodiments of the present disclosure, the modified cell is derived from any one of a somatic cell, a stem cell, and the combination thereof.

In some embodiments of the present disclosure, the modified cell is derived from an immune cell.

In some embodiments of the present disclosure, the modified cell is an animal cell or a human cell.

In some embodiments of the present disclosure, the modified cell is allogeneic with reference to the cell of the subject.

In some embodiments of the present disclosure, the unmodified cell is an immune cell, and the immune cell is any one of a T cell, Nature Killer cell, NKT cell, B cell, macrophage cell, monocyte, dendritic cell, neutrophil, and the combination thereof.

In another embodiment of the present disclosure, the unmodified cell is a stem cell, and the stem cell is a hematopoietic progenitor cell, hematopoietic stem cell, CD34⁺ cell, embryonic stem cell, a mesenchymal stem cell or iPSC cell.

In some embodiments of the present disclosure, the T cell is any one of $\alpha\beta$ T cell, $\gamma\delta$ T cell, helper T cell, regulatory cell, and the combination thereof.

In some embodiments of the present disclosure, the T cell is activated T cell.

In some embodiments of the present disclosure, the endogenous major histocompatibility

complex is a human major histocompatibility complex or a murine major histocompatibility complex.

In some embodiments of the present disclosure, the human major histocompatibility complex comprises human leukocyte antigen class I. Preferably, the gene encoding the human leukocyte antigen class I is any one or more of HLA-A, HLA-B, HLA-C, HLA-E and the combination thereof.

In some embodiments of the present disclosure, the modified cell is deficient in HLA-A and HLA-B.

In some embodiments of the present disclosure, the human major histocompatibility complex comprises human leukocyte antigen class II. Preferably, the gene encoding the human leukocyte antigen class II is any one or more of HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DQ, HLA-DR, and any combination thereof.

In some embodiments of the present disclosure, the modified cell is further deficient in human leukocyte antigen class II, *e.g.*, HLA-DR.

In some embodiments of the present disclosure, the murine major histocompatibility complex comprises histocompatibility-2 class I.

Preferably, the gene encoding the histocompatibility-2 class I is any one or more of H-2K, H-2D, and the combination thereof.

In a preferred embodiment of the present disclosure, the modified cell is further deficient in an endogenous inhibitory checkpoint molecule.

In some embodiments of the present disclosure, the modified cell is deficient in the endogenous inhibitory checkpoint molecule and the endogenous major histocompatibility complex as described in the present disclosure, and engineered to express a ligand of the endogenous inhibitory checkpoint molecule.

In some embodiments of the present disclosure, the endogenous inhibitory checkpoint molecule is any one or more of PD-1, TIM3, TIGIT, LAG3, A2AR, BTLA (CD272), CTLA-4 (CD152), IDO1, IDO2, TDO, KIR, NOX2, VISTA, SIGLEC7 (CD328), PVR (CD155), and SIGLEC9

(CD329).

In some embodiments of the present disclosure, the ligand is any one or more of PD-L1, PD-L2, HMGB1, Ceacam-1, phosphatidyl serine (PS), LSECtin, α -synuclein, FGL1, adenosine, HVEM (Herpesvirus Entry Mediator), CD28, B7-H3 (CD276), B7-H4 (VTCN1), PVR(CD155), HLA class I, sialoglycoprotein, CD112, CD113, Galectin9, CD24, and CD47.

In some embodiments of the present disclosure, the variant comprises at least one mutation that increases the capability of inducing immunoinhibitory signaling.

In some embodiments of the present disclosure, the mutation is any one of substitution, deletion, insertion, and any combination thereof.

In some embodiments of the present disclosure, the mutation is a deletion of at least one amino acid residue.

In some embodiments of the present disclosure, the variant lacks at least part of an extracellular domain, a transmembrane domain and/or an intracellular domain relative to the cognate inhibitory checkpoint molecule or the ligand thereof.

In some embodiments of the present disclosure, the inhibitory checkpoint molecule is PD-1; and/or, the ligand thereof is PD-L1.

In some embodiment of the present disclosure, the inhibitory checkpoint molecule is PD-1, and the ligand thereof is PD-L1.

In some embodiments of the present disclosure, the variant of the ligand has the amino acid sequence of SEQ ID NO: 7 or the amino acid sequence having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8% or at least 99.9% identity with SEQ ID NO: 7.

In some embodiments of the present disclosure, the modified cell is engineered to comprise a chimeric antigen receptor or a T cell receptor.

In some embodiments of the present disclosure, the CAR comprises an antigen binding domain, a transmembrane domain, and an intracellular signaling domain, further optionally the CAR

further comprises a costimulatory signaling region.

In some embodiments of the present disclosure, the antigen is one or more selected from BCMA, CD7, CD10, CD19, CD20, CD22, CD24, CD30, CD33, CD34, CD38, CD44, CD79a, CD79b, CD123, CD138, CD 179b, CEA, CLEC12A, Claudin18.2, CS-1, DLL3, EGFR, EGFRvIII, EPCAM, FLT-3, FOLR1, FOLR3, GD2, gpA33, GPC3, HER2, HM1.24, LGR5, Mesothelin, MSLN, MCSP, MICA/B, PSMA, PAMA, P-cadherin, and ROR1; *e.g.*, the antigen is CD19.

In some embodiments of the present disclosure, the modified cell or the pharmaceutical thereof is administered independently or in combination.

In some embodiments of the present disclosure, the diffuse connective tissue disease comprises systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis (RA), idiopathic inflammatory myopathy (IIM), Churg-Strauss Syndrome and microscopic polyangitis.

In some embodiments of the present disclosure, the idiopathic inflammatory myopathy (IIM) includes polymyositis (PM), dermatomyositis (DM), amyopathic dermatomyositis (ADM), immune necrotizing myopathy (IMNM), inclusion body myositis (IBM) and juvenile dermatomyositis (JDM), but not limited to.

In some embodiments of the present disclosure, the lymphatic malignancies comprises B cell lymphoma and B lymphocyte leukemia. The B cell lymphoma comprises Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL), and B lymphocyte leukemia comprises acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

In a preferred embodiment of the present disclosure, the modified cell is prepared and/or expanded *in vitro*, *in vivo* or *ex vivo*.

In some embodiments of the present disclosure, the subject in need thereof is a subject received at least 1, 2, 3 or 4 immunosuppressive therapy; preferably, the therapy is a therapy comprising one or more selected from glucocorticoids, hydroxychloroquines, mycophenolate mofetil, tacrolimus, cyclophosphamide, azathioprine, tocilizumab, methotrexate, telitacicept, belimumab and rituximab.

The second aspect of the present disclosure provides a modified cell or a population thereof,

wherein the modified cell is the modified cell defined in the first aspect.

The third aspect of the present disclosure provides a polynucleotide targeting a major histocompatibility complex, wherein the polynucleotide is capable of inactivating or weakening the expression of a major histocompatibility complex.

In some embodiments of the present disclosure, the genes encodes the major histocompatibility complex class I.

In some embodiments of the present disclosure, the polynucleotide is sgRNA.

In some embodiments of the present disclosure, the major histocompatibility complex is human major histocompatibility complex, *e.g.* HLA-A, HLA-B, HLA-C and/or HLA-E.

In some embodiments of the present disclosure, the sgRNA targets any one of the DNA sequence of SEQ ID NOs: 29-45 and 73-168.

The fourth aspect of the present disclosure provides a method for producing the modified cell of the second aspect, comprising the step of introducing the polynucleotide of the third aspect and a gene editing system.

In some embodiments of the present disclosure, the gene editing system is a CRISPR system.

The fifth aspect of the present disclosure provides a pharmaceutical composition comprising (1) the modified cell of the second aspect, and/or the polynucleotide of the third aspect, and (2) a pharmaceutically acceptable carrier and/or excipients.

The sixth aspect of the present disclosure provides a kit comprising the modified cell of the second aspect or the pharmaceutical composition of the fifth aspect.

The seventh aspect of the present disclosure provides the modified cell or a population thereof of the second aspect, the polynucleotide of third aspect, the pharmaceutical composition of the fifth aspect, and the kit of the sixth aspect for use in preventing and/or treating a condition and/or a disease of a diffuse connective tissue disease or a lymphatic malignancy.

In some embodiments of the present disclosure, the diffuse connective tissue disease comprises systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis (RA), idiopathic inflammatory myopathy (IIM), Churg-Strauss Syndrome and microscopic polyangitis.

In some embodiments of the present disclosure, the idiopathic inflammatory myopathy includes polymyositis (PM), dermatomyositis (DM), amyopathic dermatomyositis (ADM), immune necrotizing myopathy (IMNM), inclusion body myositis (IBM) and juvenile dermatomyositis (JDM), but not limited to.

In some embodiments of the present disclosure, the lymphatic malignancies comprises B cell lymphoma and B lymphocyte leukemia.

In some embodiments of the present disclosure, the B cell lymphoma comprises Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL), and B lymphocyte leukemia comprises acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

The seventh aspect of the present disclosure provides a use of the modified cell or a population thereof of the second aspect, the polynucleotide of the third aspect, the pharmaceutical composition of the fifth aspect, and the kit of the sixth aspect in preparing a medicament for preventing and/or treating a condition and/or a disease of a diffuse connective tissue disease.

In some embodiments of the present disclosure, the diffuse connective tissue disease comprises systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis (RA), idiopathic inflammatory myopathy (IIM), Churg-Strauss Syndrome and microscopic polyangitis.

In some embodiments of the present disclosure, the idiopathic inflammatory myopathy includes polymyositis (PM), dermatomyositis (DM), amyopathic dermatomyositis (ADM), immune necrotizing myopathy (IMNM), inclusion body myositis (IBM) and juvenile dermatomyositis (JDM), but not limited to.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the schematic drawing of the Chimeric Antigen Receptor (CAR) expression construct. Figure 1B shows the schematic drawing of the integration of the CAR expression construct to the lentivirus vector.

Figure 2 shows CD19 CAR expression as detected by flow cytometry in 293T cells infected with lentivirus expression of CD19 CAR-P2A-TrPD-L1, at different amount of virus (i.e. 1 μ l/well,

10 µl/well, 20 µl/well, 30 µl/well, or 50 µl/well).

Figure 3A shows CD19 CAR expression and Figure 3B shows TrPD-L1 expression, both as detected by flow cytometry in TrPDL1-CART cells transfected with lentivirus containing expression cassette for CD19 CAR-P2A-TrPD-L1.

Figure 4A and Figure 4B show cytolytic effects of TrPDL1-CART cells and TrPDL1-UCART cells on B cells (Raji cells) expressing luciferase (Raji-Luci) after incubation for 24 hours (Figure 4A) or 48 hours (Figure 4B) at different effector : target (E:T) ratios. Natural T cells were used as control.

Figure 5A and Figure 5B show release of TNF-alpha (Figure 5A) and IFN-gamma (Figure 5B) from TrPDL1-CART cells and TrPDL1-UCART cells after incubation with Raji cells expressing luciferase (Raji-Luci) for 24 hours.

Figure 6 shows schematic drawing of plasmid pELPS.

Figure 7 shows some of the sequences disclosed in the present disclosure.

Figure 8A and Figure 8B shows the therapeutic results in patients with scleroderma after treatment with CAR-T cells. The numbers of B cells and UCART cells were increased remarkably, as well as CAR copies. Meanwhile, the secretion level of cytokine IL-6 was low during the process of treatment.

Figure 9 shows the evaluation of clinical endpoints in subjects with scleroderma.

Figure 10A-Figure 10C show the changes in skin and organ functions in subjects with scleroderma. Figure 10A: Assessment of skin elasticity and stiffness by ultrasound skin elastography indicated gradual reversal of skin fibrosis. Left, elastographic color maps (red hue indicates harder tissue and blue hue indicates softer tissue). Right, elastographic measurements of skin stiffness values; Figure 10B: HRCT scans demonstrated improvement in pulmonary inflammation and fibrosis following UCART intervention in both patients; Figure 10C: CMR of the left ventricular short axis in both patients indicated a reduction in myocardial inflammation and fibrosis following UCART intervention.

Figure 11A shows the increased expansion of CAR-T cells in peripheral circulation after

injection.

Figure 11B shows the significant increase of the number of genomic CAR copies with unit mass after injection. *In vivo*, CAR T-cells are rapidly increased with 0.37% of the total circulating T cells on day 0 and 61% of the total circulating T cells on day 8, numbers of CAR T-cells were 184 and 210.1 per microliter, at day 8 and day 14 respectively. CAR copies was 63736 and 84.1 per microliter.

Figure 11C shows the distinct decrease of B cells, the target cell of the CAR-T cell of the present disclosure, in peripheral circulation after injection, and finally the number of B cells in peripheral circulation reaches zero.

Figure 12A and Figure 12B show the markedly improvement after treatment with CAR-T cells in patients suffering from dermatomyositis. The clinical indicators include LDH, CK, IgG, physical function and muscle strength.

Figure 13 shows the significantly reduction of inflammatory lesion in lungs after treatment with CAR-T cells.

Figure 14 shows the clinical assessment in Subject IMNM-01. A: Primary clinical improvement assessment; B: Additional clinical improvement assessments.

Figure 15 shows the muscle MRI scan, ILD changes, and muscle biopsy of Subject IMNM-01. A: MRI scans of the thigh muscles, including coronal and axial STIR sequences and T1-weighted images, were performed to track the extent of myositis and atrophy following UCART treatment; B: Immunohistochemistry of the muscle biopsy showed muscle damage-associated changes (enrichment of anti-SRP antibodies, CD4 and CD8 T cells, and CD68 macrophages in the microenvironment).

Figure 16 shows the evaluation of clinical endpoints in subjects with SLE.

Figure 17 shows the changes in SLEDAI score in subjects with refractory SLE.

Figure 18 shows the percentage of CART cells in lymphocytes after UCART infusion.

Figure 19 shows the CART cell count after UCART infusion.

Figure 20 shows the CAR copy number after UCART infusion.

Figure 21 shows the percentage of B cells in peripheral blood of subjects.

Figure 22 shows the B cell count in peripheral blood of subjects.

DETAILED DESCRIPTION

Definitions

As used herein, the terms or terminology are for the purpose of describing particular embodiments only and are not intended to be limiting.

It is to be noted that the term “a” or “an” entity refers to one or more of that entity; for example, “an antibody,” is understood to represent one or more antibodies. As such, the terms “a” (or “an”), “one or more,” and “at least one” can be used interchangeably herein.

The singular terms “a”, “an”, and “the” include plural referents unless context clearly indicates otherwise. By way of example, reference to “a cell” refers to one or more cells, and reference to “the method” includes reference to equivalent steps and methods disclosed herein and/or known to those skilled in the art, and so forth. Similarly, the word “and/or” is intended to include “and” and “or” unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, “*e.g.*,” is derived from the Latin *exempli gratia*, and is used herein to indicate a non-limiting example. Thus, the abbreviation “*e.g.*,” is synonymous with the term “for example”.

In all occurrences in this application where there are a series of recited numerical values, it is to be understood that any of the recited numerical values may be the upper limit or lower limit of a numerical range. It is to be further understood that the disclosure encompasses all such numerical ranges, i.e., a range having a combination of an upper numerical limit and a lower numerical limit, wherein the numerical value for each of the upper limit and the lower limit can be any numerical value recited herein. Ranges provided herein are understood to include all values within the range. For example, 1-10 is understood to include all of the values 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, and fractional values as appropriate. Similarly, ranges delimited by “at least” are understood to include the lower value provided and all higher numbers.

As used herein, “about” is understood to include within three standard deviations of the mean or within standard ranges of tolerance in the specific art. In certain embodiments, about is understood to include a variation of no more than 0.5.

The term “including” is used herein to mean, and is used interchangeably with, the phrase

“including but not limited to”. Similarly, “such as” is used herein to mean, and is used interchangeably, with the phrase “such as but not limited to”.

The term “comprising” or “comprises”, as used herein, is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the method or composition, yet open to the inclusion of unspecified elements, whether essential or not.

The term “consisting of” refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

The term “operably link” or “operably linked” refers to a juxtaposition, with or without a spacer or linker, of two or more biological sequences of interest in such a way that they are in a relationship permitting them to function in an intended manner. When used with respect to proteins, it is intended to mean that the protein sequences are linked in such a way that permits the linked product to have the intended biological function. The term may also be used with respect to polynucleotides. For instance, when a polynucleotide encoding a protein is operably linked to a regulatory sequence (*e.g.*, promoter, enhancer, silencer sequence, etc.), it is intended to mean that the polynucleotide sequences are linked in such a way that permits regulated expression of the protein from the polynucleotide.

The terms “polypeptide”, “peptide” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms also apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally-occurring amino acid, as well as to naturally-occurring amino acid polymers and non-naturally occurring amino acid polymers.

The term “nucleotide”, “nucleic acid” or “polynucleotide” as used herein includes oligonucleotides (*i.e.*, short polynucleotides). They also refer to synthetic and/or non-naturally occurring nucleic acid molecules (*e.g.*, comprising nucleotide analogues or modified backbone residues or linkages). The terms also refer to deoxyribonucleotide or ribonucleotide oligonucleotides in either single-or double-stranded form. The terms encompass nucleic acids containing analogues of natural nucleotides. The terms also encompass nucleic acid-like structures with synthetic backbones. Unless otherwise indicated, a particular polynucleotide sequence also implicitly encompasses conservatively modified variants thereof (*e.g.* degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (see Batzer *et al.*, Nucleic Acid Res. 19:5081 (1991); Ohtsuka *et al.*, J. Biol. Chem. 260:2605-2608 (1985); and Rossolini *et al.*, Mol.

Cell. Probes 8:91-98 (1994)).

“Percent (%) sequence identity” is defined as the percentage of amino acid (or nucleic acid) residues in a candidate sequence that are identical to the amino acid (or nucleic acid) residues in a reference sequence, after aligning the sequences and, if necessary, introducing gaps, to achieve the maximum number of identical amino acids (or nucleic acids). In other words, percent (%) sequence identity of an amino acid sequence (or nucleic acid sequence) can be calculated by dividing the number of amino acid residues (or bases) that are identical relative to the reference sequence to which it is being compared by the total number of the amino acid residues (or bases) in the candidate sequence or in the reference sequence, whichever is shorter. Conservative substitution of the amino acid residues is not considered as identical residues. Alignment for purposes of determining percent amino acid (or nucleic acid) sequence identity can be achieved, for example, using publicly available tools such as BLASTN, BLASTp (available on the website of U.S. National Center for Biotechnology Information (NCBI), see also, Altschul S.F. *et al*, *J. Mol. Biol.*, 215:403–410 (1990); Stephen F. *et al*, *Nucleic Acids Res.*, 25:3389–3402 (1997)), ClustalW2 (available on the website of European Bioinformatics Institute, see also, Higgins D.G. *et al*, *Methods in Enzymology*, 266:383-402 (1996); Larkin M.A. *et al*, *Bioinformatics* (Oxford, England), 23(21): 2947-8 (2007)), and ALIGN or Megalign (DNASTAR) software. Those skilled in the art may use the default parameters provided by the tool, or may customize the parameters as appropriate for the alignment, such as for example, by selecting a suitable algorithm.

A “conservative substitution” with reference to amino acid sequence refers to replacing an amino acid residue with a different amino acid residue having a side chain with similar physiochemical properties. For example, conservative substitutions can be made among amino acid residues with hydrophobic side chains (*e.g.* Met, Ala, Val, Leu, and Ile), among residues with neutral hydrophilic side chains (*e.g.* Cys, Ser, Thr, Asn and Gln), among residues with acidic side chains (*e.g.* Asp, Glu), among amino acids with basic side chains (*e.g.* His, Lys, and Arg), or among residues with aromatic side chains (*e.g.* Trp, Tyr, and Phe). As known in the art, conservative substitution usually does not cause significant change in the protein conformational structure, and therefore could retain the biological activity of a protein.

The term “functional equivalents” as used herein, refers to different forms (such as variants, fragments, fusions, derivatives and mimetics) of the parent molecule, which, despite of having difference in amino acid sequences or in chemical structures, still retains substantial biological activity of the parent molecule. The expression “retain substantial biological activity”, as used herein, means exhibiting at least part of (for example, no less than about 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%) or all of the biological activity of the parent molecule. A functional equivalent of a parent protein may include both naturally-occurring variant forms and non-

naturally occurring forms such as those obtained by recombinant methods or chemical synthesis. The functional equivalents may contain non-natural amino acid residues.

As used herein, the term “deficient” refers to insufficiency in activity or level, and can include, for example, being less than normal activity or level, or being absent or null in activity or level.

As used herein, the term “CAR”, which can be used interchangeably with the term "chimeric antigen receptor", refers to an engineered receptor or an artificial receptor or polynucleotide encoding thereof. The engineered receptor or a synthetic receptor comprises an extracellular domain that comprises an antigen binding domain, a transmembrane domain, and/or an intracellular signaling domain, which are joined one another or operably linked to each other, and confers specificity for an antigen onto an immune effector cell and bypasses MHC class I and class II restriction.

The term “chimeric antigen receptor T cell”, used interchangeably with the term “CAR-T cell” or “CART cell”, refers to a T cell or population thereof that has been engineered (*e.g.*, through genetic engineering) to express a CAR on the T cell surface. CAR-T cells can be T helper CD4⁺ and/or T effector CD8⁺ cells. CAR-T cells can bind to a target cell expressing a target antigen and initiate immune response against the target cell.

As used herein, the term “TCR”, which can be used interchangeably with the term "T cell receptor" or the term “TCR complex” refers to a natural (or endogenous) TCR or an engineered TCR. TCR refers to a disulfide-linked membrane-anchored heterodimeric protein complex normally comprising an alpha chain and a beta chain that are highly variable, that are complexed with a CD3γ, a CD3δ, and two CD3ε chains and CD3-zeta chains. The amino acid sequence of the alpha chain and beta chain varies among different T cells. The transmembrane regions of the alpha chain and the beta chain are enclosed by the CD3 transmembrane regions in an open barrel. The intracellular tails of the CD3γ, CD3δ and CD3ε molecules each contain a single conserved motif called immunoreceptor tyrosine-based activation motif (ITAM), which is critical for the signaling capacity of the TCR complex and to initiate signal transduction upon binding to antigen-MHC complex.

As used herein, the term “T cell receptor T cell”, used interchangeably with the term “TCR-T cell”, refer to a T cell or population thereof that has been engineered through biological methods (*e.g.*, genetic engineering) to express a TCR on the T cell surface. TCR-T cells can be T helper CD4⁺ and/or T effector CD8⁺ cells. TCR-T can identify MHC bound cells to initiate the immune response, and thus can target intracellular targets of the target cells.

As used herein, the term “B2M”, used interchangeable with the term "beta-2 microglobulin" refers to a protein that is a component of the class I major histocompatibility complex (MHC),

which comprises an amino acid sequence as set forth in UniProtKB Accession No. P61769 or a variant thereof that retains immunomodulatory activity, or refers to a polynucleotide encoding the protein described above. B2M is necessary for cell surface expression of MHC class I and stability of the peptide-binding groove. Few MHC class I molecules can be detected on cell surface in absence of B2M.

As used herein, the term “MHC”, used interchangeable with the term “major histocompatibility complex”, refers to a protein or a polynucleotide encoding a protein that can present an intracellular peptide on cell surface as an antigen and form complex with the antigen present on the cell surface. The complex of the MHC and antigen can interact with TCR and its co-receptors to induce immune responses (*e.g.*, T cell activation). The MHC can be categorized into MHC class I, MHC class II and MHC class III. MHC class I molecules, expressed in all nucleated cells and platelets, assists cellular immunity against intracellular pathogens (*e.g.*, viruses and bacteria). In human, MHC class I is called human leukocyte antigen (HLA) class I, which comprises HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-G molecules. MHC class II, generally occurs only on macrophages, B cells and dendritic cells, can mediate immunization to immune tolerance of an antigen under certain conditions. In human, MHC class II is called HLA class II. MHC (or HLA) molecules can act as antigens in a transplant procedure to provoke immune response in the recipient and cause transplant rejection (*Abbas AB, Lichtman AH (2009). "Ch.10 Immune responses against tumors and transplant". Basic Immunology. Functions and disorders of the immune system (3rd ed.). Saunders (Elsevier). ISBN 978-1-4160-4688-2.*

As used herein, the term “CIITA”, used interchangeably with the term “class II, major histocompatibility complex, transactivator” refers to a protein comprising an amino acid sequence that has at least about 80% sequence identity to NCBI Accession No. NP_001273331.1 or a functional fragment thereof yet retains immunomodulatory activity, or refers to a polynucleotide encoding the protein described above. CIITA is classified as a transcriptional coactivator, which functions via activating the transcription factor RFX5 to positively regulate the expression of HLA class II genes.

As used herein, the term “effector function” with respect to an immune cell refers to a specialized function of the cell, for example, the phagocytic activity, cytolytic activity or helper activity including the secretion of cytokines for a T cell.

“Treating” or “treatment” of a condition as used herein includes alleviating a condition, slowing the onset or rate of development of a condition, reducing the risk of developing a condition, preventing or delaying the development of symptoms associated with a condition, reducing or ending symptoms associated with a condition, generating a complete or partial regression of a condition, curing a condition, or some combination thereof.

The term “vector” as used herein refers to a vehicle into which a polynucleotide encoding a protein may be operably inserted so as to bring about the expression of that protein. A vector may be used to transform, transduce, or transfect a host cell so as to bring about expression of the genetic element it carries within the host cell. Examples of vectors include plasmids, phagemids, cosmids, and artificial chromosomes such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC), bacteriophages such as lambda phage or M13 phage, and animal viruses. Categories of animal viruses used as vectors include retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (*e.g.*, herpes simplex virus), poxvirus, baculovirus, papillomavirus, and papovavirus (*e.g.*, SV40). A vector may contain a variety of elements for controlling expression, including promoter sequences, transcription initiation sequences, enhancer sequences, selectable elements, and reporter genes. In addition, the vector may contain an origin of replication. A vector may also include materials to aid in its entry into the cell, including but not limited to a viral particle, a liposome, or a protein coating. A vector can be an expression vector or a cloning vector. The present disclosure provides vectors (*e.g.*, expression vectors) containing the nucleic acid sequence provided herein encoding the fusion protein, at least one promoter (*e.g.*, SV40, CMV, EF-1 α) operably linked to the nucleic acid sequence, and at least one selection marker. Examples of vectors include, but are not limited to, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (*e.g.*, herpes simplex virus), poxvirus, baculovirus, papillomavirus, papovavirus (*e.g.*, SV40), lambda phage, and M13 phage, plasmid pcDNA3.3, pMD18-T, pOptivec, pCMV, pEGFP, pIRES, pQD-Hyg-GSeu, pALTER, pBAD, pcDNA, pCal, pL, pET, pGEMEX, pGEX, pCI, pEGFT, pSV2, pFUSE, pVITRO, pVIVO, pMAL, pMONO, pSELECT, pUNO, pDUO, Psg5L, pBABE, pWPXL, pBI, p15TV-L, pPro18, pTD, pRS10, pLexA, pACT2.2, pCMV-SCRIPT.RTM., pCDM8, pCDNA1.1/amp, pcDNA3.1, pRc/RSV, PCR 2.1, pEF-1, pFB, pSG5, pXT1, pCDEF3, pSVSPORT, pEF-Bos etc.

The phrase “host cell” as used herein refers to a cell into which an exogenous polynucleotide and/or a vector has been introduced.

The term “pharmaceutically acceptable” indicates that the designated carrier, vehicle, diluent, excipient(s), and/or salt is generally chemically and/or physically compatible with the other ingredients comprising the formulation, and physiologically compatible with the recipient thereof.

The term “subject” or “individual” or “animal” or “patient” as used herein refers to human or non-human animal, including a mammal or a primate, in need of diagnosis, prognosis, amelioration, prevention and/or treatment of a disease or disorder. Mammalian subjects include humans, domestic animals, farm animals, and zoo, sports, or pet animals such as dogs, cats,

guinea pigs, rabbits, rats, mice, horses, swine, cows, bears, and so on.

I. Overview

Adoptive cell therapy involves adoptive transfer of autologous cells (*i.e.*, cells from the recipient self) or allogeneic cells (*i.e.*, cells from a donor different from the recipient). For example, immune cells, or more specifically, T cells have been of great therapeutic interest. Immune cells or T cells can be modified *ex vivo* to express CARs or TCRs on the cell surface, to mediate immune responses specifically against target cells (*e.g.* cancer cells) expressing certain target antigen.

While allogeneic cells are highly desired in clinical settings to hopefully provide for off-the-shelf cell therapy, they are also associated with risk of graft versus host disease (GVHD), due to rejection by the host's immune system through recognition of HLA class I molecules expressed on surface of the allogeneic cells.

In addition, therapeutic immune cells (such as CAR-T or TCR-T cells) can suffer from immunosuppression that results in reduced effector function of the therapeutic immune cells and hence reduced therapeutic efficacy. The immunosuppression could be induced by, for example, interaction of immune checkpoint such as PD-1 present on these immune cells with immune checkpoint ligand such as PD-L1 expressed on target cells such as tumor cells.

The present disclosure provides modified cells that, on one hand, are less susceptible to immune-mediated recognition and destruction from an allogeneic recipient, and on the other hand, maintains desirable therapeutic efficacy (*e.g.* cytotoxicity) against target cells to be treated. The disclosure will be described below in more detail.

II. Modified Cells

In one aspect, the present disclosure provides a modified cell or a population thereof, and the modified cell is, relative to an unmodified counterpart cell: i) deficient in an endogenous major histocompatibility complex; and ii) engineered to express an endogenous inhibitory checkpoint molecule or a ligand thereof. In certain embodiments, the cell is an immune cell.

In another aspect, the present disclosure provides a modified cell or a population thereof, and the modified cell is, relative to an unmodified counterpart cell: i) deficient in an endogenous inhibitory checkpoint molecule; and ii) engineered to express a ligand of the deficient endogenous inhibitory checkpoint molecule. In certain embodiments, the cell is an immune cell.

In another aspect, the present disclosure provides a modified cell or a population thereof, and the modified cell is, relative to an unmodified counterpart cell: i) deficient in an endogenous

inhibitory checkpoint molecule; ii) engineered to express a ligand of the deficient endogenous inhibitory checkpoint molecule; and iii) deficient in an endogenous major histocompatibility complex.

In order to reduce or avoid the immune rejection from the host immune system, an allogeneic immune cell can be modified to express a ligand of inhibitory checkpoint molecule (*e.g.* PD-L1), such that the expressed ligand can interact with the inhibitory checkpoint molecule (*e.g.* PD-1) expressed on the host immune cells, thereby protecting the allogeneic immune cell from immune rejection by the host immune system. However, it is unexpectedly found by the inventors that, when the modified allogeneic immune cells themselves endogenously express the inhibitory checkpoint molecule (*e.g.* PD-1), it could potentially result in self-immunosuppression, where the endogenous inhibitory checkpoint molecule present on one modified cell (*e.g.*, modified immune cell) can interact with the its ligand present on another modified cell (*e.g.*, modified immune cell). Therefore, the present inventors contemplated modified cells which, on one hand, are deficient in an endogenous inhibitory checkpoint molecule, and on the other hand, are engineered to express a ligand of the deficient endogenous inhibitory checkpoint molecule. Such modified cells are shown in the present disclosure to be useful in reducing self-immunosuppression and providing for significantly enhanced therapeutic effects.

In another aspect, the present disclosure provides a modified cell or a population thereof, which is engineered to express a ligand of an inhibitory checkpoint molecule, and the ligand is a variant of cognate ligand and the variant is characterized in having reduced capability to induce immunoinhibitory signaling relative to the cognate ligand. In certain embodiments, the variant ligand lacks a functional immunoinhibitory signaling domain of the cognate ligand.

While expression of a cognate ligand (*e.g.* PD-L1) of inhibitory checkpoint molecule on an allogeneic cell can be useful to protect from immune rejection, it is unexpectedly found by the inventors that expression of such a ligand on a cell (*e.g.* an immune cell) can also induce immunosuppression in the ligand-expressing cell, possibly through an intrinsic immunoinhibitory signaling domain of the cognate ligand. Certain ligands of inhibitory checkpoint molecule are transmembrane proteins (*e.g.* PD-L1) and are found to contain a cytoplasmic domain that can mediate immunoinhibitory signaling. Following interaction of the ligand with its corresponding inhibitory checkpoint molecule on the host immune cells, the ligand itself can also be activated in such a way that mediates immunoinhibitory signaling in the cell (*e.g.* immune cell) expressing the ligand. Accordingly, it is another aspect of the disclosure to provide modified cells engineered to express a variant ligand having reduced capability to induce immunoinhibitory signaling relative to the cognate ligand, thereby significantly reducing or minimizing the potential immune suppression on the allogenic cells expressing the ligand.

In still another aspect, the present disclosure provides a modified cell or a population thereof, wherein the modified cell is, relative to an unmodified counterpart cell: i) deficient in an endogenous inhibitory checkpoint molecule; and ii) engineered to express a ligand of the deficient endogenous inhibitory checkpoint molecule, wherein the ligand is a variant of cognate ligand and has reduced capability to induce immunoinhibitory signaling relative to the cognate ligand (for example, that lacks a functional immunoinhibitory signaling domain of the cognate ligand).

In still another aspect, the present disclosure provides a modified cell or a population thereof, wherein the modified cell is engineered to express a ligand of an inhibitory checkpoint molecule, wherein the ligand is a variant of cognate ligand and is characterized in having reduced capability to induce immunoinhibitory signaling relative to the cognate ligand, and wherein the cell is deficient in the inhibitory checkpoint molecule corresponding to the ligand.

The term “modified” as used herein with respect to cells, means that the cells are changed either structurally or functionally. For example, cells may be modified through the introduction of nucleic acids or biologically active agents.

In certain embodiments, the modified cell is an animal cell or human cell.

In certain embodiments, the modified cells are immune cells or any other types of cells that is desirable to retain cellular activity such as cytolytic activity or effector functions. In certain embodiments, the modified cell is an immune cell. As used herein, the term “immune cell” refers to a cell that is capable of eliciting an immune response against a target antigen once activated. Exemplary immune cells include, without limitation, a T cell (*e.g.*, CD4⁺ T cell, CD8⁺ T cell, cytotoxic T cell, terminal effector T cell, memory T cell, naïve T cell, regulatory T cell, natural killer T cell, gamma-delta T cell, cytokine-induced killer (CIK) T cell, and tumor infiltrating lymphocyte, a CD4⁺CD8⁺T cell, a CD4⁺CD8⁺-T cell, T helper cell (*e.g.*, T helper 1 (Th1), a T helper 2 (Th2) cell, or a helper T cell expressing CD4 (CD4⁺ T cell))), Natural Killer (NK) cell, NKT cell, B cell, macrophage cell, tumor infiltrating lymphocyte, monocyte, dendritic cell, neutrophil, and $\gamma\delta$ T cell.

Immune cells can be obtained from any location where they reside in the subject, such as blood, cord blood, thymus, pleural effusion, lymph nodes, spleen, spleen tissue, tumor and bone marrow. The isolated immune cells may be engineered directly or stored (*e.g.*, by freezing) for a period of time.

In certain embodiments, the immune cell can be a T cell, Natural Killer (NK) cell, NKT cell, B cell, macrophage cell, tumor infiltrating lymphocyte, monocyte, dendritic cell, neutrophil, or $\gamma\delta$ T cell.

In certain embodiments, the immune cell is a T cell, which is selected from the group consisting of CD4⁺ T cell, CD8⁺ T cell, cytotoxic T cell, terminal effector T cell, memory T cell, naïve T cell, regulatory T cell, natural killer T cell, gamma-delta T cell, cytokine-induced killer (CIK) T cell, and tumor infiltrating lymphocyte. In certain embodiments, the T cell is activated T cell. In some embodiments, T cells can be obtained from blood collected from a subject using various techniques known in the art, for example, apheresis. In some embodiments, CD4⁺ helper and CD8⁺ cytotoxic T cells are isolated.

In certain embodiments, the modified cell is a stem cell or a cell differentiated from the stem cell. In certain embodiments, the stem cell comprises one or more genetic modifications (*e.g.*, nucleotide insertion, deletion, and substitution) in its genome. The genetic modifications are retained and remain functional in subsequently derived cells (*e.g.*, the recombinant cells provided herein) after differentiation, expansion, passaging and/or transplantation. Detailed description about the method of engineering stem cells (*e.g.*, iPSC) and differentiating the engineered stem cells to obtain functional differentiated cells can be found in, for example, WO2021011919, disclosure of which is incorporated herein by its entirety.

In certain embodiments, the stem cell is a hematopoietic progenitor cell (*e.g.* T cell progenitor cell, NK cell progenitor cell, macrophage progenitor cell), hematopoietic stem cell (HSCs), CD34⁺ cell, a cell line of embryonic stem cell, a mesenchymal stem cell or iPSC cell.

In certain embodiments, the cell is suitable for transplantation. The cells can be any type of cells that are useful for transplantation or that are desirable to have reduced immune rejection from the host.

In one embodiment, the modified cell provided herein can be derived from cells (*e.g.* immune cells) isolated from a subject, *e.g.*, a human subject, such as a subject suspected of having a particular disease or condition, a subject who is susceptible to a particular disease or condition, or a subject who will undergo, is undergoing, or has undergone treatment for a particular disease or condition. In certain embodiments, the modified cells are derived from cells (*e.g.* immune cells isolated from a subject who is a healthy volunteer or healthy donor, or from blood bank). Hence, the modified cells provided herein can be autologous or allogeneic to the subject of interest.

In certain embodiments, the modified cell is allogeneic with reference to an intended recipient for the modified cell (*e.g.*, modified immune cell).

In certain embodiments, the cell is expanded *ex vivo*. In certain embodiments, the cell is activated prior to transplantation to an intended subject. In certain embodiments, the cell is enriched with respect to presence or absence for certain marker (*e.g.* CD3).

In certain embodiments, the present disclosure provides a population of the modified cells (*e.g.*, modified immune cells) as described above. The population of modified cells (*e.g.*, modified immune cells) as described above have one or more characteristics of the following: 1) reduced self-suppression, 2) improved effector cell function, and 3) improved cell activation and/or expansion, relative to a comparative population of counterpart cells (*e.g.*, counterpart immune cells).

In one embodiment, the modified cell provided herein has reduced immunogenicity in an allogeneic environment, and also maintains unsuppressed effector functions.

i) Deficient in an Endogenous Inhibitory Checkpoint Molecule

In certain embodiments, the modified cells are deficient in an endogenous inhibitory checkpoint molecule.

In certain embodiments, the modified cell is deficient in an endogenous inhibitory checkpoint molecule, relative to an unmodified counterpart cell. The term “counterpart cell”, as used herein, means that the cells are of the same cell type as the modified cell. The counterpart cell can be obtained from the same individual as the modified cell, or obtained from different individuals. The term “unmodified” as used herein means that the counterpart cells do not have the same modification as the modified cell, or alternatively, has no modification at all. In certain embodiments, the unmodified counterpart cell can be natural cell or native cell of the same type as the modified cell and has not been modified. In certain embodiments, the unmodified counterpart cells (*e.g.*, counterpart immune cells) are not deficient in the endogenous inhibitory checkpoint molecule. In certain embodiments, the unmodified counterpart cell is a native immune cell.

As used herein, the term “inhibitory checkpoint molecule” refers to inhibitory regulators of the immune system that has an inhibitory effect on the immune system in a host. Activation of the immune checkpoint molecule can inhibit immune response, for example, cytokine secretion, NK cell activation, T cell proliferation, and antibody production, etc. Inhibitory checkpoint molecules mediate immune escape of certain cancer cells that express inhibitory checkpoint ligands of such inhibitory checkpoint molecules.

In certain embodiments, the inhibitory checkpoint molecule is endogenous to the unmodified cell. “Endogenous” as used herein with respect to a protein, means that the protein is natively or naturally expressed in a cell.

Exemplary inhibitory checkpoint molecules include, without limitation, PD-1, TIM3, TIGIT, LAG3, A2AR, BTLA (CD272), CTLA-4 (CD152), IDO1, IDO2, TDO, KIR, NOX2, VISTA, SIGLEC7 (CD328), PVR(CD155), and SIGLEC9 (CD329).

“PD-1” is short for Programmed Death 1 (PD-1) receptor. Exemplary sequence of human PD-1 includes human PD-1 protein and human PD-1 gene. More detailed description can be found in, for example, Philips et al., (January 1, 2015). "Therapeutic uses of anti-PD-1 and anti-PD-L1 antibodies". *Int Immunol.* 27 (1): 39-46.

“TIM3” is short for T-cell Immunoglobulin domain and Mucin domain 3, and more detailed description can be found in, for example, Zhu et al., (August 11, 2010). TIM-3 and its regulatory role in immune responses. *Curr Top Microbiol Immunol. Current Topics in Microbiology and Immunology.* 350. pp. 1-15.

“TIGIT” is short for T cell immunoreceptor with Ig and ITIM domains, and more detailed description can be found in, for example, Yu et al., (Jan 2009). "The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells". *Nat Immunol.* 10 (1): 48-57. doi:10.1038/ni.1674.

“LAG3” is short for Lymphocyte Activation Gene-3, and more detailed description can be found in, for example, Huang et al., (October 1, 2004). "Role of LAG-3 in regulatory T cells". *Immunity.* 21 (4): :503–13; Grosso et al. (November 1, 2007). "LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems". *J Clin Invest.* 117 (11): 3383–92.

“A2AR” is short for Adenosine A2A receptor, and more detailed description can be found in, for example, Leone et al., (April 8, 2015). "A2aR antagonists: Next generation checkpoint blockade for cancer immunotherapy", *Comput Struct Biotechnol J.* 13: 265–72.

“BTLA” is short for B and T Lymphocyte Attenuator and is used interchangeably with the term “CD272”, and more detailed description can be found in, for example, Derré et al., (January 1, 2010). BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination". *J Clin Invest.* 120 (1): 157–67.

“CTLA-4” is short for Cytotoxic T-Lymphocyte-Associated protein 4 and also called CD152, and more detailed description can be found in, for example, Kolar et al., (January 1, 2009). "CTLA-4 (CD152) controls homeostasis and suppressive capacity of regulatory T cells in mice". *Arthritis Rheum.* 60 (1): 123–32.

“IDO1” and “IDO2” are short for Indoleamine 2,3-dioxygenase 1 and Indoleamine 2,3-dioxygenase 2 respectively, and more detailed description can be found in, for example, Prendergast et al., (July 1, 2014). "Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer". *Cancer Immunol Immunother.* 63 (7): 721–35.

“TDO” is short for tryptophan 2,3-dioxygenase.

“KIR” is short for Killer-cell Immunoglobulin-like Receptor, which is a receptor for MHC Class I molecules on Natural Killer cells.

“NOX2” is short for nicotinamide adenine dinucleotide phosphate NADPH oxidase isoform 2, and more detailed description can be found in, for example, Martner et al., (October 1, 2018). "NOX2 in autoimmunity, tumor growth and metastasis". J Pathol. 247 (2): 151–154.

“VISTA” is short for V-domain Ig suppressor of T cell activation, and more detailed description can be found in, for example, Wang et al., (March 14, 2011). "VISTA, a novel mouse Ig superfamily ligand that negatively regulates T cell responses". J Exp Med. 208 (3): 577–92 and Lines et al., (April 1, 2014). "VISTA is an immune checkpoint molecule for human T cells". Cancer Res. 74 (7): 1924–32.

PVR (CD155) is short for poliovirus receptor and is a member of the nectin-like family of proteins. PVR is a member of an immunoglobulin superfamily defined by the presence of immunoglobulin domains V, a C1-like domain and a C2 domain in the extracellular region.

“SIGLEC7” is short for Sialic acid-binding immunoglobulin-type lectin 7, also designated as CD328. “SIGLEC9” is short for Sialic acid-binding immunoglobulin-type lectin 9, also designated as CD329, and more detailed description can be found in, for example, Varki et al., (2007). "Siglecs and their roles in the immune system". Nature Reviews Immunology. 7 (4): 255–266.

In certain embodiments, the inhibitory checkpoint molecule is selected from a group consisting of PD-1, TIM3, TIGIT, LAG3, A2AR, BTLA (CD272), CTLA-4 (CD152), IDO1, IDO2, TDO, KIR, NOX2, VISTA, SIGLEC7 (CD328), PVR (CD155), and SIGLEC9 (CD329).

In certain embodiments, the cell is T cell and the endogenous inhibitory checkpoint molecule is selected from the group consisting of: PD-1, TIM3, TIGIT, LAG3, A2AR, BTLA (CD272), CTLA-4 (CD152), IDO1, IDO2, TDO, NOX2, VISTA, SIGLEC7 (CD328), PVR (CD155) and SIGLEC9 (CD329).

In certain embodiments, the cell is NK cell and the endogenous inhibitory checkpoint molecule is selected from the group consisting of: PD-1, TIM3, TIGIT, LAG3, A2AR, BTLA (CD272), IDO1, IDO2, KIR, TDO, NOX2, VISTA, SIGLEC7 (CD328), PVR(CD155) and SIGLEC9 (CD329).

In certain embodiments, the cell is DC cell and the endogenous inhibitory checkpoint molecule is selected from the group consisting of: A2AR, CD47, IDO1, IDO2, and TDO.

In certain embodiments, the cell is macrophage and the endogenous inhibitory checkpoint molecule is selected from the group consisting of: A2AR, CD47, IDO1, IDO2, and CD24.

The term “deficient” as used herein means that the endogenous inhibitory checkpoint molecule in the modified cell is either absent or null in activity or level, or is less than normal activity or level relative to that in an unmodified counterpart cell. For example, the gene of the endogenous inhibitory checkpoint molecule in the modified cell can be deleted or mutated to render a loss-of-function phenotype. For another example, the endogenous inhibitory checkpoint molecule can be expressed in the modified cell at a reduced level, or in a form that has reduced activity. The level and/activity of the endogenous inhibitory checkpoint molecule can be measured using various techniques known in the art, such as immunohistochemistry, western blot, immunofluorescence microscopy, and quantitative flow cytometry.

Any suitable methods can be used to modify a cell to be deficient in the endogenous inhibitory checkpoint molecule, for example, by gene editing, by interfering with expression of the endogenous inhibitory checkpoint molecule, or by promoting degradation of endogenous inhibitory checkpoint molecule or its encoding mRNA. In certain embodiments, the modified cell (*e.g.*, modified immune cell) comprises a first mutation within a coding sequence or a regulatory sequence within a genomic region of the inhibitory checkpoint molecule, wherein the mutation reduces expression or activity of the inhibitory checkpoint molecule. In certain embodiments, the modified cell (*e.g.*, modified immune cell) comprises a first interfering oligonucleotide targeting mRNA of the inhibitory checkpoint molecule, wherein the interfering oligonucleotide reduces expression of the inhibitory checkpoint molecule. In certain embodiments, the modified cell (*e.g.*, modified immune cell) comprises an introduced protein, polypeptide or small molecule that inhibits the expression and/or activity of the inhibitory checkpoint molecule.

In certain embodiments, the inhibitory checkpoint molecule is PD-1. In certain embodiments, the modified cells are deficient in endogenous PD-1.

The level of PD-1 can be measured by methods known in the art such as immunohistochemistry, western blot, immunofluorescence microscopy, and quantitative flow cytometry.

The activity of PD-1 (*i.e.*, PD-1 mediated immuno-inhibition) can be determined by measuring the expression level of the activated version of PD-1 (*e.g.*, phosphorylated PD-1, such as PD-1 with phosphorylated Y248, which is indispensable for delivery of PD-1 inhibitory function (Kankana et al., Phosphorylation of PD-1-Y248 is a marker of PD-1-mediated inhibitory function in human T cells, *Scientific Reports* (2019) 9:17252)). The activity of PD-1 may also be measured by measuring the expression level of active version of a protein downstream PD-1 that contributes to the inhibitory function of PD-1 in immune cells, such as phosphorylated phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG) (Marianne et al., Transmembrane adaptor protein PAG is a mediator of PD-1 inhibitory signaling in human

T cells, COMMUNICATIONS BIOLOGY (2021) 4:672).

ii) Engineered to Express A Ligand of the Inhibitory Checkpoint Molecule

In certain embodiments, the modified cell provided herein is engineered to express a ligand of an inhibitory checkpoint molecule.

In certain embodiments, the modified cell provided herein is engineered to express a ligand of the deficient endogenous inhibitory checkpoint molecule provided herein.

As used herein, the term “ligand” with respect to the inhibitory checkpoint molecule refers to a molecule that is capable of binding to and activating the inhibitory checkpoint molecule to exert immune suppression effects. In certain embodiments, the ligand comprises a transmembrane protein comprising an extracellular domain, a transmembrane domain and an intracellular domain.

The ligand can be a cognate ligand of the inhibitory checkpoint molecule, or alternatively can be a variant of the cognate ligand, or alternatively can be any agonist of the inhibitory checkpoint molecule. The term “cognate ligand” as used herein refers to an endogenous ligand or natural ligand of a protein.

Any suitable methods can be used to engineer a cell to express a ligand of the inhibitory checkpoint molecule. In certain embodiments, the modified cell comprises a first exogenous polynucleotide comprising a coding sequence for the ligand. In certain embodiments, the first exogenous polynucleotide further comprises a coding sequence for a signal peptide operably linked to the coding sequence for the ligand. In certain embodiments, the first exogenous polynucleotide further comprises a promoter operably linked to the coding sequence for the ligand. In certain embodiments, the first exogenous polynucleotide is mRNA, or an expression vector, optionally a viral expression vector.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) has introduced expression of the ligand. The term “introduced expression” with respect to the ligand, as used herein, refers expression of the ligand by providing a nucleic acid (*e.g.*, coding sequence) for the ligand into a cell. The nucleic acid can be incorporated into the genome of the cell or can be transiently provided to the cell.

In certain embodiments, the ligand is selected from a group consisting of PD-L1, PD-L2, HMGB1, Ceacam-1, phosphatidyl serine (PS), LSECtin, α -synuclein, FGL1, adenosine, HVEM (Herpesvirus Entry Mediator), CD28, B7-H3 (CD276), B7-H4 (VTCN1), PVR(CD155), HLA class I, sialoglycoprotein, CD112, CD113, Galectin9, CD24, and CD47. These ligands are known in the art and the encoding genes are available from public database such as Gene

database of National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine, for example under the respective Gene ID: PD-L1 (Gene ID: 29126), PD-L2 (Gene ID: 80380), HMGB1 (Gene ID: 3146), Ceacam-1 (Gene ID: 634), LSECtin (Gene ID: 339390), a-synuclein (Gene ID: 6622), FGL1 (Gene ID: 2267), HVEM (Herpesvirus Entry Mediator) (Gene ID: 8764), CD28 (Gene ID: 940), B7-H3 (CD276) (Gene ID: 80381), B7-H4 (VTCN1) (Gene ID: 79679), PVR(CD155) (Gene ID: 5817), HLA class I, CD112 (Gene ID: 5819), CD113 (Gene ID: 25945), Galectin9 (Gene ID: 16859), CD24 (Gene ID: 100133941), and CD47 (Gene ID: 961).

a) Expressed Ligand is Paired with the Deficient Endogenous Inhibitory Checkpoint Molecule

In certain embodiments, the modified cells provided herein are deficient in an endogenous inhibitory checkpoint molecule, and meanwhile engineered to express a ligand of such endogenous inhibitory checkpoint molecule. In other words, the deficient inhibitory checkpoint molecule and the expressed ligand are paired.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is PD-1, and the expressed ligand comprises at least one of PD-L1 or PD-L2 or a functional equivalent thereof or a variant thereof.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is TIM3, and the expressed ligand comprises at least one of galectin9, HMGB1, Ceacam-1 or phosphatidyl serine (PS), or a functional equivalent thereof capable of binding to TIM3.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is LAG-3, and the expressed ligand comprises at least one of galectin-3, LSECtin, a-synuclein, or FGL1 or a functional equivalent thereof capable of binding to LAG-3.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is TIGIT, and the expressed ligand comprises at least one of CD155, CD113 or CD112 or a functional equivalent thereof capable of binding to TIGIT.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is Adenosine A2A receptor (A2AR), and the ligand comprises adenosine.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is BTLA, and the expressed ligand comprises Herpesvirus Entry Mediator (HVEM) or a functional equivalent thereof capable of binding to BTLA.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is CTLA-4, and the expressed ligand comprises CD28 or a functional equivalent thereof capable of binding to CTLA4.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is Killer-cell Immunoglobulin-like Receptor (KIR), and the expressed ligand comprises HLA class I or a functional equivalent thereof capable of binding to KIR.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is VISTA (V-domain Ig suppressor of T cell activation), and the expressed ligand comprises at least one of PD-L1 or PD-L2 or a functional equivalent thereof capable of binding to VISTA.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is Sialic acid-binding immunoglobulin-type lectin 7 (SIGLEC-7, CD328), and the ligand comprises sialoglycoprotein or a functional equivalent thereof capable of binding to SIGLEC-7.

In certain embodiments, the deficient endogenous inhibitory checkpoint molecule is SIGLEC-9 (CD329), and the ligand comprises sialoglycoprotein or a functional equivalent thereof capable of binding to SIGLEC-9.

In certain embodiments, the modified cell comprises in its genome a first mutation within a coding sequence or a regulatory sequence of the inhibitory checkpoint molecule, and/or an exogenous first polynucleotide comprising a coding sequence for a ligand of the inhibitory checkpoint molecule, wherein the first mutation reduces expression or activity of the inhibitory checkpoint molecule, and the exogenous first polynucleotide results in introduced expression of the ligand of the inhibitory checkpoint molecule.

In certain embodiments, the cell is further deficient in one or more additional endogenous inhibitory checkpoint molecule.

In certain embodiments, when the inhibitory checkpoint molecule is PD-1, the ligand can be PD-L1 or PD-L2 or functional equivalents thereof. Binding of PD-L1 or PD-L2 or functional equivalents thereof to PD-1 recruits Src homology 2 domain containing phosphatases 1 and 2 (SHP-1/SHP-2) to the tyrosine based switch motif (ITSM) of PD-1, which leads to dephosphorylation of signaling kinases such as CD3 ζ , PKC θ and ZAP70 and results in a global inhibitory action of T cell expansion (Yokosuka et al., Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. J Exp Med 2012;209:1201–17).

The ligands as described above may be screened by generating a library of mutants of the ligand and testing the binding affinity of the mutants to its inhibitory checkpoint molecule, as well as the intrinsic immunoinhibitory signaling of the mutants. Those mutants that retain high binding affinity to their inhibitory checkpoint molecule yet show reduced intrinsic immunoinhibitory signaling would be selected as the ligand of the modified cells (*e.g.*, immune cells) of the present disclosure. The intrinsic immunoinhibitory signaling can be measured by various techniques

known in the art, for example, FACS, PCR, Western Blot, or by detecting the expression of intracellular signaling proteins or modifications thereof.

In certain embodiments, the ligand comprises a secreted agonist or cell surface agonist that is capable of activating the inhibitory checkpoint molecule. For example, a PD-1 agonist can be capable of binding to PD-1 for induction of PD-1-mediated inhibitory signaling, and therefore can be engineered to be released from or expressed on the surface of the modified cell (*e.g.*, modified immune cell). Exemplary PD-1 agonists include, without limitation, a PD-1 binding protein or miniproteins that can be computationally designed and can interact with PD-1 and inhibit T cell activation (Cassie et al., Computational design of a synthetic PD-1 agonist. PNAS July 20, 2021 118 (29) e2102164118). These PD-1 agonists can be engineered onto the surface of the modified cell (*e.g.*, modified immune cell) by protein engineering techniques, for example, fusing the PD-1 agonists to a transmembrane domain and other elements required for expression of the PD-1 agonists outside the cell surface while being anchored to the surface.

b) Variant of Ligand of the Inhibitory Checkpoint Molecule

In certain embodiments, the ligand provided herein is a variant of cognate ligand of the endogenous inhibitory checkpoint molecule.

As used herein, the term “variant” of a ligand refers to a protein different from but homologous to the cognate or native ligand, and substantially retaining the capability of the cognate or native ligand to bind to the binding partner (*e.g.* the immune checkpoint molecule). A variant may differ from the parent peptide by one or more amino acid residues. For example, a variant may have conservative substitutions, deletions, or insertions of one or more amino acid residues of the parent protein. In certain embodiments, the variant has at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to the cognate ligand, yet substantially retaining the capability of the cognate ligand to bind to its binding partner (*e.g.* the immune checkpoint molecule). A “variant” of a natural or native ligand (*e.g.* native PD-L1) can be different forms, including without limitation, fragments, mutants, fusions or any combination thereof, of the naturally occurring or native ligand. A variant ligand does not contain the full length of the native ligand. However, the variant may contain a fragment or portion of the native ligand, or a fusion protein comprising such a fragment or portion. A variant ligand may also comprise a mutated form of the full-length native ligand or a mutated form of a fragment of the native ligand, or a fusion protein comprising such a mutated form.

The variant ligand has reduced capability to induce immunoinhibitory signaling relative to the cognate ligand.

It is found that at least some cognate ligands of inhibitory checkpoint molecules have signaling

domains that could mediate immunoinhibitory signaling. Following interaction of the cognate ligand with its corresponding inhibitory checkpoint molecule, the cognate ligand itself can also be activated in such a way that mediates immunoinhibitory signaling in the modified cell that expresses the cognate ligand. Without wishing to be bound by any theory, it is contemplated that mutation or deletion of the immunoinhibitory signaling domain(s) of the cognate ligand would reduce the “back-signaling” in the modified cell expressing the ligand (*e.g.*, modified immune cell). Upon binding of the ligand to its corresponding inhibitory checkpoint molecule (*e.g.* those present on the immune cells of the recipient). This could be helpful to reduce the inhibitory signaling in the modified cell (*e.g.*, modified immune cell) that would otherwise compromise the effector function of the modified cell (*e.g.*, modified immune cell), and accordingly increase the effector function of the modified cell (*e.g.*, modified immune cell).

In certain embodiments, the variant ligand has reduced immunoinhibitory signaling relative to the cognate ligand. In certain embodiments, the immunoinhibitory signaling of the variant is reduced such that it is substantially lower than (for example, at least 20% lower than, at least 40% lower than, at least 60% lower than or at least 80% lower than) the normal or baseline level of the immunoinhibitory signaling of the cognate ligand.

In certain embodiments, the modified cells provided herein are engineered to express a ligand of an inhibitory checkpoint molecule, wherein the ligand is a variant of cognate ligand and lacks a functional immunoinhibitory signaling domain of the cognate ligand.

In certain embodiments, the variant comprises at least one mutation that reduces capability to induce immunoinhibitory signaling. In certain embodiments, the at least one mutation comprises a substitution, deletion, insertion, or any combination thereof.

In certain embodiments, the at least one mutation comprises deletion of at least 1 amino acid residue, for example, deletion of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, at least 50 amino acid residues, or more.

In certain embodiments, the cognate ligand comprises a transmembrane protein comprising an extracellular domain, a transmembrane domain and an intracellular domain. In certain embodiments, the at least one mutation is within the intracellular domain of the cognate ligand, or within the transmembrane domain of cognate ligand, or within the extracellular domain of cognate ligand, or any combination thereof.

In certain embodiments, the immunoinhibitory signaling domain is within the intracellular domain or the transmembrane domain of the cognate ligand. For example, some ligands of inhibitory checkpoint molecules are transmembrane proteins (*e.g.* PD-L1) and are found to contain a cytoplasmic domain that can mediate immunoinhibitory signaling.

In certain embodiments, the variant is a variant of cognate ligand of immunoinhibitory checkpoint molecule, wherein the cognate ligand is selected from the group consisting of PD-L1, PD-L2, B7-H3 (CD276), B7-H4 (VTCN1), PVR(CD155), HLA class I, sialoglycoprotein, CD112, CD113, Galectin9, CD24, and CD47, wherein the variant have reduced capability to induce immunoinhibitory signaling relative to the cognate ligand, and/or lack a functional immunoinhibitory signaling domain of its corresponding cognate ligand.

Cells can be engineered to express the variant ligand of the inhibitory checkpoint molecule using methods described above, that is, by introducing to the modified cell the first exogenous polynucleotide comprising a coding sequence for the ligand. In certain embodiments, the first exogenous polynucleotide comprises a coding sequence for the variant ligand. In certain embodiments, the first exogenous polynucleotide further comprises a coding sequence for a signal peptide operably linked to the coding sequence for the variant ligand. In certain embodiments, the first exogenous polynucleotide further comprises a promoter operably linked to the coding sequence for the variant ligand. In certain embodiments, the first exogenous polynucleotide is mRNA, or an expression vector, optionally a viral expression vector. In certain embodiments, the modified cell comprises the first exogenous polynucleotide comprising a coding sequence for the variant ligand.

c) PD-L1 variant

In certain embodiments, the variant ligand of the inhibitory checkpoint molecule provided herein is a PD-L1 variant or a PD-L2 variant.

PD-L1 is a type I transmembrane protein with a size of ~40kDa and consists of IgV-like and IgC-like extracellular domains, a hydrophobic transmembrane domain and a short cytoplasmic tail made from 30-31 amino acids. (Kythreotou et al., PD-L1. *J. Clin. Pathol.* 71, 189–194 (2018); Dong et al., B7-H1, a third member of the B7 family, costimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 1999;5:1365–9; and Chen et al., Regulation of PD-L1: a novel role of pro-survival signalling in cancer. *Ann Oncol* 2016;27:409–16.). PD-L1 is encoded by the PDCDL1 gene that is found on chromosome 9 in humans at position p24.1 (NCBI gene resource CD274 molecule [Homo sapiens (human)]-gene-NCBI. 2017 <https://www.ncbi.nlm.nih.gov/gene> (accessed 29 Jun 2017).). The full length of PD-L1 is encoded by seven exons, which corresponds to a protein of 290 amino acids, including its signal peptide. Recent studies found that PD-L1 engagement induced T cell apoptosis by inducing intracellular signaling that is

similar to that of PD-1 upon activation, which was named as “back-signaling” (Brian et al., PD-L1 engagement on T cells promotes self-tolerance and suppression of neighboring macrophages and effector T cells in cancer, *Nature Immunology* | VOL 21 442 | April 2020 | 442–454).

In certain embodiments, the PD-L1 variant or the PD-L2 variant has reduced capability to induce immunoinhibitory signaling, yet substantially retaining the capability to bind to PD-1 or binding affinity to PD-1.

The binding capacity and/or affinity of the PD-L1 variant or PD-L2 variant can be measured by any suitable assays such as ELISA, Western Blot, flow cytometry assay, and other binding assay. In certain embodiments, the binding capacity and/or affinity is measured by flow cytometry. In general, PD-L1-expressing cells can be incubated with a range of concentrations of PD-1-expressing cells or soluble PD-L1 (e.g. PD-1 extracellular domain, optionally fused with Fc), followed by incubation with a fluorescently labelled secondary antibody, and then analyzed for fluorescent signal intensity.

The immunoinhibitory signaling of PD-L1 variant or the PD-L2 variant can be determined by any suitable assays. For example, PD-1 knockout (PD-1^{-/-}) T cells can be engineered to express PD-L1 or a PD-L1 variant provided herein, and the resultant engineered T cell can be incubated with PD-1 expressing cell or soluble PD-1 (e.g. PD-1 extracellular domain, optionally fused with Fc), followed by detection of T cell activation or proliferation or differentiation. When the engineered T cells express full-length PD-L1, PD-1 engagement can inhibit activation or proliferation or differentiation of the engineered T cells, while engineered T cells expressing the PD-L1 variant provided herein shows much less inhibition by PD-1 engagement.

In certain embodiments, the PD-L1 variant or the PD-L2 variant provided herein has no more than 80%, 70%, 60%, 50%, 40%, 30%, 20% or 10% or 5% of the immunoinhibitory signaling of the full length wild-type PD-L1. In certain embodiments, the PD-L1 variant or the PD-L2 variant provided herein has undetectable immunoinhibitory signaling.

In certain embodiments, the PD-L1 variant lacks a functional immunoinhibitory signaling domain of the native PD-L1. In certain embodiments, the PD-L2 variant lacks a functional immunoinhibitory signaling domain of the native PD-L2. In certain embodiments, the PD-L1 variant does not contain full length PD-L1. The PD-L1 variant does not contain full-length of native PD-L1. The PD-L2 variant does not contain full-length of native PD-L2. In certain embodiments, the PD-L1 variant comprises a truncated native PD-L1 or a truncated mutant PD-L1. In certain embodiments, the truncated variant comprises a C-terminal truncated PD-L1.

In certain embodiments, the PD-L1 variant comprises at least one mutation that reduces capability to induce immunoinhibitory signaling, relative to native PD-L1. In certain

embodiments, the PD-L1 variant comprises at least one mutation within the intracellular domain (*e.g.* SEQ ID NO: 1), or within the transmembrane domain (*e.g.* SEQ ID NO: 2), or within the extracellular domain (*e.g.* SEQ ID NO: 3), or any combination thereof. In certain embodiments, the PD-L1 variant comprises at least one mutation within the domain spanning the extracellular domain and the intracellular domain, for example, a domain comprising or consisting of an amino acid sequence of SEQ ID NO: 5. In other embodiments, the at least one mutation comprises the mutations outside the one or more domains involved in transducing immunoinhibitory signaling yet results in configurational changes that interrupt the transduction of immunoinhibitory signaling.

In certain embodiments, the PD-L1 variant comprises deletion of at least part of the intracellular domain of PD-L1 (*e.g.* SEQ ID NO: 1), or deletion of at least part of the transmembrane domain of PD-L1 (*e.g.* SEQ ID NO: 2), or deletion of at least part of the extracellular domain of PD-L1 (*e.g.* SEQ ID NO: 3), or any combination thereof. In certain embodiments, the PD-L1 variant comprises deletion of at least part of the domain spanning the extracellular domain and the intracellular domain, for example, a domain comprising or consisting of an amino acid sequence of SEQ ID NO: 5.

In certain embodiments, the PD-L1 variant comprises a deletion of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, or at least 31 amino acid residues, within a region corresponding to the amino acid sequence of SEQ ID NO: 1 of the native PD-L1.

In certain embodiments, the engineered intracellular domain and/or the engineered transmembrane domain of PD-L1 comprises a deletion of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, or at least 21 amino acid residues, within a region spanning from position 1 to 21 of SEQ ID NO: 2, within a region corresponding to the amino acid sequence of SEQ ID NO: 2 of the native PD-L1.

In certain embodiments, the PD-L1 variant comprises a deletion of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, at least 50 amino acid residues, or at least 51 amino

acid residues within a region corresponding to the amino acid sequence of SEQ ID NO: 5 of the native PD-L1.

In certain embodiments, the PD-L1 variant is a C-terminal truncation variant that has a C-terminal deletion.

In certain embodiments, the PD-L1 variant (*e.g.* C-terminal truncation variant) comprises an amino acid sequence at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 100% identical to the native PD-L1 (*e.g.* SEQ ID NO: 11) or to an equal length portion of SEQ ID NO: 11, yet substantially retaining the capability to bind to PD-1. The PD-L1 variant does not comprise an amino acid sequence of SEQ ID NO: 11.

In certain embodiments, the PD-L1 variant (*e.g.* C-terminal truncation variant) comprises an amino acid sequence at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 100% identical to the native PD-L1 (*e.g.* SEQ ID NO: 7) or to an equal length portion of SEQ ID NO: 7, yet substantially retaining the capability to bind to PD-1.

In certain embodiments, the PD-L1 variant comprises a truncated PD-L1 (*e.g.* C-terminal truncated PD-L1). A truncated PD-L1 can be a truncated version of native PD-L1, or a truncated version of mutant PD-L1. In certain embodiments, the truncated PD-L1 has a length of no more than 271, no more than 270, no more than 265, no more than 260, no more than 255, no more than 250, no more than 245, no more than 240, no more than 235, no more than 230, no more than 225, no more than 220 amino acid residues (*e.g.* from 220 to 272, from 220 to 271, from 220 to 270, from 220 to 269, from 220 to 268, from 220 to 267, from 220 to 265, from 220 to 264, from 220 to 263, from 220 to 262, from 220 to 261, from 220 to 260, from 220 to 259, from 220 to 258, from 220 to 257, from 220 to 256, from 220 to 255, from 220 to 254, from 220 to 253, from 220 to 252, from 220 to 251, from 220 to 250, from 220 to 249, from 220 to 248, from 220 to 247, from 220 to 246, from 220 to 245, from 220 to 244, from 220 to 243, from 220 to 242, from 220 to 241, from 241 to 272, from 241 to 271, from 241 to 270, from 241 to 269, from 241 to 268, from 241 to 267, from 241 to 265, from 241 to 264, from 241 to 263, from 241 to 262, from 241 to 261, from 241 to 260, from 241 to 259, from 241 to 258, from 241 to 257, from 241 to 256, from 241 to 255, from 241 to 254, from 241 to 253, from 241 to 252, from 241 to 251, from 241 to 250, from 241 to 249, from 241 to 248, from 241 to 247, from 241 to 246, from 241 to 245, from 241 to 244, from 241 to 243, from 241 to 242 amino acid residues).

In certain embodiments, the PD-L1 variant comprises an amino acid sequence of SEQ ID NO: 7. In certain embodiments, the amino acid sequence of the PD-L1 variant is SEQ ID NO: 7.

Cells can be engineered to express the PD-L1 variant using methods described above, that is, by

introducing to the modified cell the first exogenous polynucleotide comprising a coding sequence for the PD-L1 variant. In certain embodiments, the first exogenous polynucleotide comprises a coding sequence for the PD-L1 variant. In certain embodiments, the first exogenous polynucleotide further comprises a coding sequence for a signal peptide operably linked to the coding sequence for the PD-L1 variant. In certain embodiments, the signal peptide comprises an amino acid sequence of SEQ ID NO: 4. In certain embodiments, the first exogenous polynucleotide further comprises a promoter operably linked to the coding sequence for the PD-L1 variant. In certain embodiments, the first exogenous polynucleotide is mRNA, or an expression vector, optionally a viral expression vector.

In certain embodiments, the modified cell comprises the first exogenous polynucleotide comprising a coding sequence for the PD-L1 variant.

d) Polynucleotides Encoding PD-L1 Variant, Cells Comprising the Polynucleotides

In a separate aspect, the present disclosure further provides a protein comprising a PD-L1 variant having reduced capability to induce immunoinhibitory signaling relative to native PD-L1. In certain embodiments, the PD-L1 variant lacks a functional immunoinhibitory signaling domain of native PD-L1. In certain embodiments, the PD-L1 variant comprises at least one mutation that reduces capability to induce immunoinhibitory signaling. The other aspects of the PD-L1 variant are same those as described in the above sections.

In certain embodiments, the protein comprises the PD-L1 variant which is linked to a polypeptide of interest. In certain embodiments, the polypeptide of interest can be expressed on a cell surface or can be secreted. In certain embodiments, the polypeptide of interest can bind to a target antigen (*e.g.* a target cell antigen) provided herein. In certain embodiments, the polypeptide of interest is a chimeric antigen receptor (CAR) or an engineered TCR or other cell surface receptor or ligand. More details of polypeptide of interest, CAR and engineered TCR are described below and applicable in the embodiments here.

In certain embodiments, the PD-L1 variant is linked to the polypeptide of interest via a linker. In certain embodiments, the linker is cleavable. In certain embodiments, the cleavable linker is a self-cleaving peptide, such as 2A peptides, such as P2A (*e.g.* SEQ ID NO: 16), T2A, or F2A. In certain embodiments, the nucleotide sequence encoding P2A has a nucleotide sequence of SEQ ID NO: 15.

In certain embodiments, the protein further comprises a signal peptide. In certain embodiments, the PD-L1 variant further comprises a signal peptide.

In certain embodiments, the protein comprises the PD-L1 variant linked to a linker, optionally a cleavable linker. In certain embodiments, the protein comprises the PD-L1 variant linked to

P2A. In certain embodiments, the protein comprises an amino acid sequence of SEQ ID NO: 17.

In certain embodiments, the present disclosure further provides a polynucleotide encoding the protein provided herein. In certain embodiments, such polynucleotide has a nucleotide sequence comprising SEQ ID NO: 13, 14, or 18, or a homologous sequence thereof having at least 50% sequence identity, optionally encoding the same protein or encoding a protein variant having at least 90% or 95% or 99% sequence identity.

In certain embodiments, the present disclosure further provides an expression vector comprising the polynucleotide provided herein.

In certain embodiments, the present disclosure further provides a recombinant cell comprising the expression vector provided herein. In certain embodiments, the recombinant cell is an animal cell or human cell. In certain embodiments, the recombinant cell is an immune cell. In certain embodiments, the recombinant cell is a stem cell or a cell differentiated from the stem cell. In certain embodiments, the cell is suitable for transplantation. In certain embodiments, the recombinant cell is allogeneic with reference to an intended recipient for the recombinant cell.

In another aspect, the present disclosure provides a recombinant cell or a population thereof, which is engineered to express a PD-L1 variant or a PD-L2 variant.

In another aspect, the present disclosure also provides a population of recombinant cells produced ex vivo by the method as described above. In certain embodiments, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the population of cells express a detectable level of the PD-L1 variant provided herein.

In another aspect, the present disclosure also provides a method of producing a recombinant cell with increased immunotolerance in an allogeneic host, comprising introducing to the cell the expression vector provided herein under conditions suitable for expression of the polynucleotide provided herein in the cell, thereby increasing immunotolerance of the recombinant cell. As used herein, the term “immunotolerance” refers to the ability of tolerating or reducing the immune rejection of the recombinant cell by the immune system of an allogeneic host. In certain embodiments, the recombinant cell is further deficient in antigen-presenting proteins such as MHC I class protein (HLA class I) and/or MHC II class protein (HLA class II).

iii) Expression of Other Polypeptides of Interest

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof provided herein further expresses another polypeptide of interest. In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof provided herein has been modified, or is further modified, to introduce a second exogenous polynucleotide comprising a

coding sequence for a polypeptide of interest, and optionally the modified cell expresses the polypeptide of interest.

In certain embodiments, the polypeptide of interest can be expressed on the cell surface or can be secreted. In certain embodiments, the polypeptide of interest can bind to a target antigen such as a target cell antigen.

A target cell antigen may be a cell surface marker on a target cell associated with a disease or condition of interest. In certain embodiments, the target cell antigen comprises a tumor antigen, an inflammation-associated antigen, or an infectious agent associated antigen (*e.g.* viral antigen). In certain embodiments, the target cell antigen is associated with a disease selected from the group consisting of a cancer, an autoimmune disease, or an infectious disease.

Examples of tumor antigen include, without limitation, BCMA, CD7, CD10, CD19, CD20, CD22, CD24, CD30, CD33, CD34, CD38, CD44, CD79a, CD79b, CD123, CD138, CD 179b, CEA, CLEC12A, Claudin18.2, CS-I, DLL3, EGFR, EGFRvIII, EPCAM, FLT-3, FOLR1, FOLR3, GD2, gpA33, GPC3, HER2, HM1.24, LGR5, Mesothelin, MSLN, MCSP, MICA/B, PSMA, PAMA, P-cadherin, and ROR1.

Any suitable methods can be used to engineer a cell to further express the polypeptide of interest. In certain embodiments, the modified cell comprises a second exogenous polynucleotide comprising a coding sequence for the other polypeptide of interest. In certain embodiments, the second exogenous polynucleotide further comprises a coding sequence for a signal peptide operably linked to the coding sequence for the other polypeptide of interest. In certain embodiments, the second exogenous polynucleotide further comprises a promoter operably linked to the coding sequence for the other polypeptide of interest. In certain embodiments, the second exogenous polynucleotide is mRNA, or an expression vector, optionally a viral expression vector.

In certain embodiments, the other polypeptide of interest is co-expressed with the ligand of the endogenous inhibitory checkpoint molecule. In certain embodiments, the other polypeptide of interest is expressed as a fusion protein with the ligand or the variant ligand. In certain embodiments, the other polypeptide of interest is linked to the ligand (or the variant of cognate ligand) of the endogenous inhibitory checkpoint molecule, optionally via a linker, for example, a cleavable linker.

In certain embodiments, the cleavable linker is a self-cleaving peptide, such as 2A peptides. 2A peptides are viral oligopeptides having a length of 18–22 amino acid residues, and mediate cleavage of polypeptides during translation in eukaryotic cells. Different viral 2A peptides have generally been named after the virus they were derived from, for example, F2A is derived from

foot-and-mouth disease virus, E2A from equine rhinitis A virus, P2A from porcine teschovirus-1 2A, and T2A from thogotovirus 2A (see for details in, Liu, Z et al, Scientific Reports, volume 7, Article number: 2193 (2017)). A highly conserved sequence GDVEXNPGP (SEQ ID NO: 6, wherein X can be any amino acid residue) is shared by different 2As at the C-terminus, and is essential for the creation of steric hindrance and ribosome skipping. 2A peptides are useful to induce high levels of downstream protein expression for multi-gene co-expression, and they are small in size thus bearing a lower risk of interfering with the function of co-expressed genes. 2A peptides have also been successfully employed for polycistronic and bi-cistronic multigene expression. In certain embodiments, the cleavable linker comprises P2A (*e.g.* SEQ ID NO: 16), F2A (*e.g.* SEQ ID NO: 9, GSGVKQTLNFDLLKLAGDVESNPGP), T2A (*e.g.* SEQ ID NO: 71, GSGEGRGSLTTCGDVEENPGP) and E2A (*e.g.* SEQ ID NO: 72, GSGQCTNYALLKLAGDVESNPGP). In certain embodiments, the nucleotide sequence encoding P2A has a nucleotide sequence of SEQ ID NO: 15.

In such embodiments, the second exogenous polynucleotide and the first exogenous polynucleotide are in one combined polynucleotide which encodes the fusion protein comprising the polypeptide of interest and the ligand (*e.g.* variant of cognate ligand) of the endogenous inhibitory checkpoint molecule. The combined polynucleotide may further comprise a coding sequence for a signal peptide operably linked to the coding sequence for the fusion protein. In certain embodiments, combined polynucleotide may further comprise a promoter operably linked to the coding sequence for the fusion protein.

In certain embodiments, the polypeptide of interest is a chimeric antigen receptor (CAR) or an engineered TCR or other cell surface receptor or ligand.

In certain embodiments, the CAR or the engineered TCR is capable of binding to the target cell antigen such as a target cell surface antigen as described above.

In certain embodiments, the modified cell is a modified T cell, and optionally the modified T cell is further deficient in endogenous T cell receptor. Details are described below sections in more detail.

a) Chimeric Antigen Receptor (CAR)

Chimeric antigen receptors (CARs) are engineered chimeric receptors that combine an antigen-binding domain with one or more signaling domains for immune cell activation. Upon binding to its target antigen, CAR can mediate antigen-specific cellular immune activity in the modified immune cells (*e.g.* T cells and NK cells), enabling such CAR-expressing immune cells to eliminate cells (*e.g.* tumor cells) expressing the target antigen. Cellular immune activity mediated by CAR can also include proliferation of the CAR-expressing immune cells, release of

cytotoxic factors, *e.g.* perforin, granzymes, and granulysin, and initiation of cytolysis and/or apoptosis of the target cells.

In certain embodiments, the CAR comprises an antigen binding domain, a transmembrane domain, and an intracellular signaling domain. In certain embodiments, the intracellular signaling domain comprises a TCR signaling domain.

In certain embodiments, the CAR further comprises a costimulatory signaling region. The costimulatory signaling region and the intracellular signaling domain can be linked to each other in a random or in a specified order, optionally with a short peptide linker having a suitable length, such as for example, between 2 and 10 amino acids (*e.g.* glycine-serine doublet linker).

In some embodiments, the antigen binding domain of the CAR comprises one or more antigen-binding fragment derived from an antibody against a target antigen. In some embodiments, it is beneficial for the antigen binding domain to be derived from the same species in which the CAR will ultimately be used in. For example, for use in humans, it may be beneficial to have the antigen binding domain used in the CAR derived from a human antibody or a humanized antibody. In some embodiments, the antigen binding domain comprises a single chain variable fragment (scFv). In some embodiment, the antigen binding domain may exist in a variety of other forms including, for example, Fv, Fab, and (Fab')₂, as well as bi-functional (*i.e.* bi-specific) hybrid antibody fragments (*e.g.*, Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)). In certain embodiments, the antigen binding domain comprises a Fab or a scFv.

In certain embodiments, the CAR comprises a transmembrane domain fused to the extracellular antigen-binding domain of the CAR. In one embodiment, the transmembrane domain can be selected such that it is naturally associated with one of the domains in the CAR. In some instances, the transmembrane domain can be selected or modified to avoid binding to transmembrane domains of other members of the T cell receptor complex.

The transmembrane domain of the CAR provided herein may be derived from transmembrane domains of any natural membrane-bound or transmembrane protein, such as, for example, the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, and CD154. In some embodiments, the transmembrane domain of the CAR can also use a variety of human hinges such as human Ig (immunoglobulin) hinge. In certain embodiments, the CAR further comprises a hinge region of an antibody. For example, the hinge region may comprise an amino acid sequence of SEQ ID NO: 25. In certain embodiments, the CAR further comprises a CD8 α transmembrane domain (TM), which optionally comprises an amino acid sequence of SEQ ID NO: 26.

Alternatively, the transmembrane domain of the CAR provided herein may be synthetic, for example, comprising predominantly hydrophobic residues such as leucine and valine. In one embodiment, a triplet of phenylalanine, tryptophan and valine is included at each end of a synthetic transmembrane domain. Optionally, a short oligo- or polypeptide linker, between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the intracellular signaling domain of the CAR. A glycine-serine doublet provides a particularly suitable linker.

The intracellular signaling domain of the CAR can activate the immune cell which expresses the CAR, to exert at least one of the normal effector functions of the immune cell, for example, cytolytic activity or helper activity including the secretion of cytokines. The intracellular signaling domain can be either full-length of a natural intracellular signal transduction domain, or a fragment thereof sufficient to transduce the effector function signal.

Exemplary intracellular signaling domains useful in the CARs provided herein include, the cytoplasmic sequences of the T cell receptor (TCR) and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant of these sequences and any synthetic sequence that has the same functional capability.

In certain embodiments, the intracellular signaling domain comprises a TCR signaling domain. The TCR signaling domain that acts in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs. Examples of ITAM containing TCR signaling domains useful in the CAR provided herein include those derived from TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d. In certain embodiments, the TCR signaling domain comprises a cytoplasmic signaling sequence derived from CD3-zeta. In certain embodiments, the CAR further comprises a CD3 ζ signal transduction domain comprising an amino acid sequence SEQ ID NO: 28.

In certain embodiments, the CAR provided herein further comprises a co-stimulatory signaling region. Co-stimulatory signaling region acts in an antigen-independent manner to mediate CAR signaling or activation, and can be derived from a co-stimulatory molecule required for an efficient response of lymphocytes to an antigen. Exemplary co-stimulatory molecules include, CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83, and the like. In certain embodiments, the CAR further comprises a 4-1BB costimulatory signal transduction region comprising an amino acid sequence of SEQ ID NO: 27.

An example of CAR include, for example, a single-chain variable fragment (scFv) derived from a monoclonal antibody fused to CD3-zeta transmembrane and endodomain. Such CARs result

in the transmission of a zeta signal in response to specific binding of scFv to its target. Methods of preparing CARs are publicly available (see, *e.g.*, Grupp et al., N Engl J Med., 368:1509-1518, 2013; Park et al., Trends Biotechnol., 29:550-557, 2011; Haso et al., (2013) Blood, 121, 1165-1174; Han et al., J. Hematol Oncol.6:47, 2013; WO2012/079000; U.S. Pub.2012/0213783; and WO2013/059593, each of which is incorporated by reference herein in its entirety). Detailed description about CAR can also be found in, for example, Cheng et al., Engineering CAR-T cells. Biomarker Research (2017) 5:22; Yang et al., Challenges and Opportunities of Allogeneic Donor-Derived CAR T cells. Curr Opin Hematol. 2015 November; 22(6): 509–515.

In certain embodiments, the CAR comprises an anti-CD19 scFv. The anti-CD19 scFv is derived from CD19 antibody FMC63 comprising a light chain variable region comprising an amino acid sequence of SEQ ID NO: 22, and a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 24, optionally linked via a linker (*e.g.* comprising an amino acid sequence of SEQ ID NO: 23).

b) Engineered T cell receptor (TCR)

In certain embodiments, the engineered TCR comprises an engineered antigen binding domain. In certain embodiments, the engineered TCR may have higher affinity for a target cell surface antigen than a wildtype TCR. In other embodiments, the TCR may include a modified chain, such as a modified alpha or beta chain. Such modifications may include, but are not limited to, N-deglycosylation, altered domain (such as an engineered variable region to target a specific antigen or increase affinity), addition of one or more disulfide bonds, entire or fragment of a chain derived from a different species, and any combination thereof.

Techniques for engineering and expressing TCR are known in the art. For example, TCR can be produced as TCR heterodimers including the native disulphide bond that associate the respective subunits, see, *e.g.*, Garboczi, et al., (1996), Nature 384(6605): 134-41; Garboczi, et al., (1996), J Immunol 157(12): 5403-10; Chang et al., (1994), PNAS USA 91: 11408-11412; Davodeau et al., (1993), J. Biol. Chem.268(21): 15455-15460; Golden et al., (1997), J. Imm. Meth.206: 163-169; U.S. Pat. No. 6,080,840. Detailed description about engineered TCR can also be found in, for example, Ping et al., T-cell receptor-engineered T cells for cancer treatment: current status and future directions. Protein Cell 2018, 9(3):254–266.

iv) Modified Cell Further Deficient in Antigen Presentation Proteins and Other Proteins

In certain embodiments, the modified cells (*e.g.*, modified immune cells) provided herein are further deficient in one or more proteins involved in antigen processing, antigen presentation, antigen recognition, and/or antigen response, including but not limited, beta-2-microglobulin (B2M), class II major histocompatibility complex transactivator (CIITA), and ICP47 polypeptide.

In certain embodiments, the modified cells (*e.g.*, modified immune cells) provided herein are further deficient in HLA class I protein, or deficient in HLA class II protein, or deficient in both.

In certain embodiments, the modified cell is further deficient in HLA-A, HLA-B, HLA-C, or any combination thereof. In certain embodiments, the modified cell is further deficient in both HLA-A and HLA-B.

Human Leukocyte Antigen (HLA) is one of the major makers for the immune system to recognize “self” antigens versus “non-self” antigens (Hudson et al., Leukocyte Ig-like receptors – a model for MHC class I disease associations. *Front Immunol* (2016) 7:281. doi:10.3389/fimmu.2016.00281). During allograft, HLA class I molecules are the major antigens that induce host versus graft disease (HVGD). Engineering or knocking down the HLA class I molecules on the allogeneic cells can reduce the immunogenicity of the allogeneic cells, see, for example, WO2021050601, WO2021062227, WO2021011919, disclosure of which are incorporated herein by their entirety.

HLA class I molecules are heterodimers consisting of a highly variable α chain and a constant β chain, wherein the constant beta chain is also called as β microglobulin (B2M). The α chain and the β chain of HLA class I molecules bind to an antigenic peptide inside a cell for assembly and the assembled HLA-antigenic peptide complex is transported onto the cell surface. The antigenic peptide to be loaded onto HLA class I molecules are generated in cytosol and transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) (Michalek et al., (1993) A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. *Nature* 363(6429): 552–554.). In the ER, the antigenic peptides can be further trimmed by the aminopeptidases ERAP1 and ERAP2 (Saveanu et al., (2005) Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. *Nat Immunol* 6(7): 689–697.). Accordingly, deficient in α chain or the β chain of HLA class I molecules or the proteins involved in the assembly of HLA to peptides (*e.g.*, TAP proteins) would negatively affect the assembly of the HLA class I molecules to the antigenic peptide and its cell surface expression. In addition, dysregulation of proteins of the antigen-processing machinery (APM), such as tapasin, would also lead to low quantity of HLA class I cell surface expression (Camilla et al., HLA class I is most tightly linked to levels of tapasin compared with other antigen-processing proteins in glioblastoma. *British Journal of Cancer* (2015) 113, 952–962).

In certain embodiments, the immune cell is further deficient in B2M.

Any suitable methods can be used to modify a cell to be deficient in HLA class I protein, for example, by gene editing, by interfering with expression of the HLA class I protein, or by promoting degradation of HLA class I protein or its encoding mRNA. In certain embodiments,

the modified cell (*e.g.*, modified immune cell) comprises a second mutation within a coding sequence or a regulatory sequence within a genomic region of the HLA class I protein or B2M, wherein the mutation (*e.g.*, deletion, substitution, insertion) reduces expression or activity of the HLA class I protein or B2M. In certain embodiments, the modified cell (*e.g.*, modified immune cell) comprises a second interfering oligonucleotide targeting mRNA of the HLA class I or B2M, thereby causing the HLA class I protein or B2M to have reduced expression therein.

In certain embodiments, the modified cells (*e.g.*, modified immune cells) provided herein are further deficient in HLA class II protein. In certain embodiments, the immune cell is further deficient in CIITA.

Any suitable methods can be used to modify a cell to be deficient in HLA class II protein, for example, by gene editing, by interfering with expression of the HLA class II protein, or by promoting degradation of HLA class II protein or its encoding mRNA. In certain embodiments, the modified cell (*e.g.*, modified immune cell) comprises a third mutation within a coding sequence or a regulatory sequence within a genomic region of the HLA class II protein or CIITA, wherein the mutation (*e.g.*, deletion, substitution, insertion) reduces expression or activity of the HLA class II protein or CIITA. In certain embodiments, the modified cell (*e.g.*, modified immune cell) comprises a third interfering oligonucleotide targeting mRNA of the HLA class II protein or CIITA, thereby causing the HLA class II protein or CIITA to have reduced expression therein.

In certain embodiments, the modified cells (*e.g.*, modified immune cells, modified T cells) provided herein are further deficient in endogenous T cell receptor.

By “deficient in endogenous T cell receptor”, it is meant that the level of endogenous TCR or the number of endogenous TCR molecules present on a modified cell (*e.g.* a modified T cell) would not induce or would induce less than 10% functions (*e.g.*, proliferation, produce cytokines, and/or induce lytic activity, etc.) of the T cell even at a high immunogen dose (for example, a dose that is near saturating for the induction of the aforementioned functions of a T cell in a subject receiving the immunogen dose). The level of TCR or the number of TCR molecules present on a T cell can be measured by methods, such as quantitative flow cytometry, immunofluorescence microscopy and optical trap, as described in Davis et al., (1998). Determination of CD4 antigen density on cells: role of antibody valency, avidity, clones, and conjugation. Cytometry 33, 197–205; Labrecque et al., How much TCR does a T cell need? Immunity. 2001; 15:71–82 and James et al., Mathematical modeling of chimeric TCR triggering predicts the magnitude of target lysis and its impairment by TCR downmodulation. Journal of Immunology. 2010; 184:4284–94; Wei et al., (1999). Mapping the sensitivity of T cells with an optical trap: polarity and minimal number of receptors for Ca(2+) signaling. Proc. Natl. Acad. Sci. 96, 8471–8476. For example, studies have shown that about 1000 or less (*e.g.*, 200-400, 450)

surface TCR molecules were needed for T cells to respond to immunization *in vivo* at a high immunogen dose in mice (Labrecque et al., How much TCR does a T cell need? *Immunity*, 2001; 15:71–82; Wei et al., (1999). Mapping the sensitivity of T cells with an optical trap: polarity and minimal number of receptors for Ca(2+) signaling. *Proc. Natl. Acad. Sci.* 96, 8471–8476).

In certain embodiments, the modified cells (*e.g.*, modified immune cells, modified T cells) provided herein are further deficient in T cell receptor alpha chain constant region (TRAC), T cell receptor beta constant 1 (TRBC1), T cell receptor beta constant 2 (TRBC2), or any combination thereof.

Any suitable methods can be used to modify a cell to be deficient in endogenous TCR, for example, by gene editing, by interfering with expression of the endogenous TCR, or by promoting degradation of endogenous TCR or its encoding mRNA. In certain embodiments, the modified cell (*e.g.*, modified immune cell) comprises a fourth mutation within a coding sequence or a regulatory sequence within a genomic region of the endogenous TCR or TRAC or TRBC1 or TRBC2 or any combination thereof, wherein the mutation (*e.g.*, deletion, substitution, insertion) reduces expression or activity of the endogenous TCR or TRAC or TRBC1 or TRBC2 or any combination thereof. In certain embodiments, the modified cell (*e.g.*, modified immune cell) comprises a fourth interfering oligonucleotide targeting mRNA of the endogenous TCR or TRAC or TRBC1 or TRBC2 or any combination thereof, thereby causing the endogenous TCR or TRAC or TRBC1 or TRBC2 or any combination thereof to have reduced expression therein.

In certain embodiments, the second mutation, or the third mutation, or the fourth mutation or any combination thereof is introduced by any suitable means known in the art, for example without limitation, gene editing. Suitable methods are described in more detail in below sections of the present disclosure.

v) Combinations of Modifications in the Modified Cell

In certain embodiments, the modified cells provided herein or a population thereof are modified to be deficient in a combination of molecules, for example, a combination of endogenous immunoinhibitory checkpoint molecule and HLA-I class protein; or a combination of endogenous immunoinhibitory checkpoint molecule and HLA-II class protein; or a combination of endogenous immunoinhibitory checkpoint molecule, HLA-I class protein and HLA class II protein. In certain embodiments, when the modified cells are T cells, they are further modified to be deficient in endogenous T cell receptor.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express ligand of the deficient immunoinhibitory checkpoint molecule.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof

is deficient in HLA-A and HLA-B relative to an unmodified counterpart cell.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is deficient in HLA-A, HLA-B and CIITA relative to an unmodified counterpart cell.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is deficient in B2M, CIITA and PD-1 relative to an unmodified counterpart cell. In certain of these embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express PD-L1 or PD-L2 relative to an unmodified counterpart cell. In certain embodiments, the PD-L1 is a PD-L1 variant provided herein. In certain embodiments, the PD-L2 is a PD-L2 variant.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is deficient in B2M, CIITA and TIM-3 relative to an unmodified counterpart cell. In certain of these embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express a ligand of TIM3 (*e.g.* Galectin9) relative to an unmodified counterpart cell. In certain embodiments, the ligand of TIM3 9 (*e.g.* Galectin9) is a variant having reduced immunoinhibitory signaling relative to native ligand (*e.g.* native Galectin9).

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is deficient in B2M, CIITA and LAG-3 relative to an unmodified counterpart cell. In certain of these embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express a ligand of LAG3 (*e.g.* galectin-3, LSECtin, a-synuclein, FGL1 or MHCII) relative to an unmodified counterpart cell. In certain embodiments, the ligand of LAG3 is a variant having reduced immunoinhibitory signaling relative to native ligand of LAG3.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is deficient in B2M, CIITA and TIGIT relative to an unmodified counterpart cell. In certain of these embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express a ligand of TIGIT (*e.g.* CD155, CD112, or CD113) relative to an unmodified counterpart cell. In certain embodiments, the ligand of TIGIT is a variant having reduced immunoinhibitory signaling relative to native ligand of TIGIT.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is deficient in HLA-A, HLA-B, CIITA and PD-1 relative to an unmodified counterpart cell. In certain of these embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express PD-L1 or PD-L2 relative to an unmodified counterpart cell. In certain embodiments, the PD-L1 is a PD-L1 variant provided herein. In certain embodiments, the PD-L2 is a PD-L2 variant.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof

is deficient in HLA-A, HLA-B, CIITA and TIM3 relative to an unmodified counterpart cell. In certain of these embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express a ligand of TIM3 9 (*e.g.* Galectin9) relative to an unmodified counterpart cell. In certain embodiments, the ligand of TIM3 9 (*e.g.* Galectin9) is a variant having reduced immunoinhibitory signaling relative to native ligand (*e.g.* native Galectin9).

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is deficient in HLA-A, HLA-B, CIITA and LAG3 relative to an unmodified counterpart cell. In certain of these embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express a ligand of LAG3 (*e.g.* galectin-3, LSECtin, a-synuclein, FGL1 or MHCII) relative to an unmodified counterpart cell. In certain embodiments, the ligand of LAG3 is a variant having reduced immunoinhibitory signaling relative to native ligand of LAG3.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is deficient in HLA-A, HLA-B, CIITA and TIGIT relative to an unmodified counterpart cell. In certain of these embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express a ligand of TIGIT (*e.g.* CD155, CD112, or CD113) relative to an unmodified counterpart cell. In certain embodiments, the ligand of TIGIT is a variant having reduced immunoinhibitory signaling relative to native ligand of TIGIT.

In certain embodiments, the modified cell (*e.g.*, modified immune cell) or a population thereof is further engineered to express a polypeptide of interest (*e.g.* a CAR or an engineered TCR or other cell surface receptor or ligand).

Method of Producing Modified cell (e.g., modified immune cell)

Also provided herein are methods of producing the modified cell (*e.g.*, modified immune cell) as described above, comprising: providing a starting cell; modifying the starting cell to reduce expression or activity of the endogenous inhibitory checkpoint molecule and to increase expression or activity of the ligand of the endogenous inhibitory checkpoint molecule, thereby obtaining the modified cell (*e.g.*, modified immune cell). The endogenous inhibitory checkpoint molecule and ligands thereof could be same as described in the sections above. In certain embodiments, the starting cell is an immune cell, for example, an immune cell expressing the endogenous inhibitory checkpoint molecule. In certain embodiments, the starting cell is an immune cell, for example, an immune cell expressing both the endogenous inhibitory checkpoint molecule and the ligand to the endogenous inhibitory checkpoint molecule.

Also provided herein are methods of producing the modified cell (*e.g.*, modified immune cell)

as described above, comprising: providing a starting cell (*e.g.*, immune cell) expressing the endogenous inhibitory checkpoint and the ligand to the endogenous inhibitory checkpoint molecule; and modifying the starting cell (*e.g.*, immune cell) to reduce expression or activity of the endogenous inhibitory checkpoint molecule, thereby obtaining the modified cell (*e.g.*, modified immune cell). The endogenous inhibitory checkpoint molecule and ligands thereof could be same as described in the sections above.

Also provided herein are methods of producing the modified cell (*e.g.*, modified immune cell) as described above, comprising: providing a starting cell (*e.g.*, immune cell) deficient in the endogenous inhibitory checkpoint molecule; and modifying the starting cell (*e.g.*, immune cell) to increase expression or activity of the ligand to the endogenous inhibitory checkpoint molecule, thereby obtaining the modified cell (*e.g.*, modified immune cell). The endogenous inhibitory checkpoint molecule and ligands thereof could be same as described in the sections above.

Also provided herein are methods of producing the modified cell (*e.g.*, modified immune cell) as described above, comprising: providing a starting cell; modifying the starting cell to express a ligand of an inhibitory checkpoint molecule, wherein the ligand is a variant of cognate ligand having reduced capability to induce immunoinhibitory signaling relative to the cognate ligand. The variant of cognate ligand could be same as described in the sections above.

In certain embodiments, the step of modifying the starting cell (*e.g.*, immune cell) to reduce expression or activity of the endogenous inhibitory checkpoint molecule comprises: a) introducing to the starting cells a first mutation to a coding sequence or a regulatory sequence within a genomic region of the inhibitory checkpoint molecule, thereby causing the inhibitory checkpoint molecule to have reduced expression or reduced activity therein; or b) introducing to the starting cell (*e.g.*, immune cell) a first interfering oligonucleotide targeting mRNA of the inhibitory checkpoint molecule, thereby causing the inhibitory checkpoint molecule to have reduced expression therein; or c) introducing to the starting cell (*e.g.*, immune cell) a protein, polypeptide or small molecule that inhibits the expression and/or activity of the inhibitory checkpoint molecule; or d) contacting the starting cell (*e.g.*, immune cell) with a protein, polypeptide or small molecule that inhibits the expression and/or activity of the inhibitory checkpoint molecule.

In certain embodiments, the method may further comprise a step of modifying the starting cell (*e.g.*, immune cell), or the cell modified from the starting cell, to reduce expression or activity of the HLA class I protein, which step comprises: a) introducing to the starting cell, or the cell modified from the starting cell, a second mutation to a coding sequence or a regulatory sequence within a genomic region of the HLA class I protein (*e.g.* HLA-A, HLA-B, or B2M), thereby causing the HLA class I protein to have reduced expression or reduced activity therein; or b)

introducing to the starting cell (*e.g.*, immune cell), or the cell modified from the starting cell, a second interfering oligonucleotide targeting mRNA of the HLA class I protein (*e.g.* HLA-A, HLA-B, or B2M), thereby causing the HLA class I protein to have reduced expression therein; or c) introducing to the starting cell (*e.g.*, immune cell) or the cell modified from the starting cell, a protein, polypeptide or small molecule that inhibits the expression and/or activity of the HLA class I protein (*e.g.* HLA-A, HLA-B, or B2M); or d) contacting the starting cell (*e.g.*, immune cell), or the cell modified from the starting cell, with a protein, polypeptide or small molecule that inhibits the expression and/or activity of the HLA class I protein.

In certain embodiments, the method may further comprise a step of modifying the starting cell (*e.g.*, immune cell), or the cell modified from the starting cell, to reduce expression or activity of the HLA class II protein, which step comprises: a) introducing to the starting cell, or the cell modified from the starting cell, a third mutation to a coding sequence or a regulatory sequence within a genomic region of the HLA class II protein (*e.g.* CIITA), thereby causing the HLA class II protein to have reduced expression or reduced activity therein; or b) introducing to the starting cell (*e.g.*, immune cell), or the cell modified from the starting cell, a second interfering oligonucleotide targeting mRNA of the HLA class II protein (*e.g.* CIITA), thereby causing the HLA class II protein to have reduced expression therein; or c) introducing to the starting cell (*e.g.*, immune cell) or the cell modified from the starting cell, a protein, polypeptide or small molecule that inhibits the expression and/or activity of the HLA class II protein (*e.g.* CIITA); or d) contacting the starting cell (*e.g.*, immune cell), or the cell modified from the starting cell, with a protein, polypeptide or small molecule that inhibits the expression and/or activity of the HLA class II protein.

In certain embodiments, the method may further comprise a step of modifying the starting cell (*e.g.*, immune cell), or the cell modified from the starting cell, to reduce expression or activity of the endogenous TCR, which step comprises: a) introducing to the starting cell, or the cell modified from the starting cell, a fourth mutation to a coding sequence or a regulatory sequence within a genomic region of the endogenous TCR (*e.g.* TRAC or TRBC1 or TRBC2), thereby causing the endogenous TCR to have reduced expression or reduced activity therein; or b) introducing to the starting cell (*e.g.*, immune cell), or the cell modified from the starting cell, a third interfering oligonucleotide targeting mRNA of the endogenous TCR (*e.g.* TRAC or TRBC1 or TRBC2), thereby causing the endogenous TCR to have reduced expression therein; or c) introducing to the starting cell (*e.g.*, immune cell) or the cell modified from the starting cell, a protein, polypeptide or small molecule that inhibits the expression and/or activity of the endogenous TCR; or d) contacting the starting cell (*e.g.*, immune cell), or the cell modified from the starting cell, with a protein, polypeptide or small molecule that inhibits the expression and/or activity of the endogenous TCR.

In certain embodiments, the first mutation, the second mutation, the third mutation and/or the fourth mutation is introduced by means of gene editing. Many nucleic acid based composition and methods are useful for gene editing, such as for example, antisense RNA, antigomer RNA, siRNA, shRNA, and a CRISPR system.

In certain embodiments, the gene editing comprises: introducing to the immune cells i) a sequence-targeting protein, or a polynucleotide encoding the same, and ii) an oligonucleotide that is complementary to a target sequence in the coding sequence or a regulatory sequence within a genomic region of the target molecule (*e.g.* the inhibitory checkpoint molecule, the HLA class I protein and/or the HLA class II protein, or the endogenous TCR). In certain embodiments, the sequence-targeting protein comprises a sequence-guided DNA endonuclease, such as Cas9, T7, Cas3, Cas8a, Cas8b, Cas10d, Cse1, Csy1, Csn2, Cas4, Cas10, Csm2, Cmr5, and Fok1.

In certain embodiments, the oligonucleotide comprises a guide nucleic acid. The guide nucleic acid sequence includes a RNA sequence, a DNA sequence, a combination thereof (a RNA-DNA combination sequence), or a sequence with synthetic nucleotides. The guide nucleic acid sequence can be a single molecule or a double molecule. In one embodiment, the guide nucleic acid sequence comprises a single guide RNA. In one embodiment, the guide nucleic acid sequence is at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 or more nucleotides in length.

In certain embodiments, the oligonucleotide comprises one or more guide nucleic acids (*e.g.* guide RNA) targeting: a) a coding sequence or a regulatory sequence within a genomic region of the HLA class I protein (*e.g.* HLA-A, HLA-B, or B2M); b) a coding sequence or a regulatory sequence within a genomic region of the HLA class II protein (*e.g.* CIITA); c) a coding sequence or a regulatory sequence within a genomic region of the inhibitory checkpoint molecule (*e.g.* PD-1, TIM3, LAG3, or TIGIT), or d) a coding sequence or a regulatory sequence within a genomic region of an endogenous TCR (*e.g.* TRAC or TRBC1 or TRBC2).

In certain embodiments, the oligonucleotide comprises one or more guide nucleic acids (*e.g.* guide RNA) targeting a genomic region of HLA-A, and is complementary to a fragment of SEQ ID NO: 63 or 64.

In certain embodiments, the oligonucleotide comprises one or more guide nucleic acids (*e.g.* guide RNA) targeting a genomic region of HLA-B, and is complementary to a fragment of SEQ ID NO: 65 or 66.

In certain embodiments, the oligonucleotide comprises one or more guide nucleic acids (*e.g.* guide RNA) targeting a genomic region of B2M, and is complementary to a fragment of SEQ ID NO: 67.

In certain embodiments, the oligonucleotide comprises one or more guide nucleic acids (*e.g.* guide RNA) targeting a genomic region of CIITA, and is complementary to a fragment of SEQ ID NO: 68.

In certain embodiments, the oligonucleotide comprises one or more guide nucleic acids (*e.g.* guide RNA) targeting a genomic region of PD-1, and is complementary to a fragment of SEQ ID NO: 69.

In certain embodiments, the oligonucleotide comprises one or more guide nucleic acids (*e.g.* guide RNA) targeting a genomic region of TRAC, and is complementary to a fragment of SEQ ID NO: 70.

In certain embodiments, the step of modifying the starting cell (*e.g.*, immune cell) to increase expression or activity of the ligand of the inhibitory checkpoint molecule comprises: a) introducing to the starting cell (*e.g.*, immune cell) an exogenous first polynucleotide comprising a coding sequence for the ligand, thereby causing expression of the ligand therefrom; b) knocking out or knocking down one or more suppressive transcription factors; c) editing one or more regulatory sequences for expression of the gene encoding the ligand; or d) knocking in the gene encoding the ligand. For example, Keisuke et al., found that truncation of the 3'-untranslated (3'-UTR) region of PD-L1 transcripts leads to elevated expression of PD-L1 in various cancers (Keisuke et al. Aberrant PD-L1 expression through 3'-UTR disruption in multiple cancers. *Nature*. 2016 Jun 16;534(7607):402-6.). Accordingly, it can be expected that the modified immune cell that comprises truncation in the 3'-UTR region of PD-L1 transcripts would result in increased expression of PD-L1. Methods of disrupting the 3'-UTR region of PD-L1 transcripts can be found, for example, in Keisuke et al. Aberrant PD-L1 expression through 3'-UTR disruption in multiple cancers. *Nature*. 2016 Jun 16;534(7607):402-6.

In certain embodiments, the step of modifying the starting cell (*e.g.*, immune cell) to express the variant of cognate ligand comprises, a) introducing to the starting cell (*e.g.*, immune cell) a first exogenous polynucleotide comprising a coding sequence for the variant, thereby causing expression of the variant therefrom; or b) knocking in the gene encoding the variant.

In certain embodiments, the first exogenous polynucleotide further comprises a coding sequence for a signal peptide operably linked to the coding sequence for the ligand or for the variant of the ligand.

In certain embodiments, further comprises a coding sequence for a signal peptide operably linked to the coding sequence for the ligand or for the variant of the ligand.

In certain embodiments, the first exogenous polynucleotide further comprises a promoter operably linked to the coding sequence for the ligand.

In certain embodiments, the exogenous first polynucleotide is an mRNA, an expression vector, optionally a retroviral vector, a lentiviral vector, an adenoviral vector, an adeno-associated viral (AAV) vector, a virus-like particle (VLP), a herpes simplex virus (HSV) vector, a plasmid, a minicircle, a nanoplasmid, a DNA vector, or an RNA vector. In certain embodiments, the expression vector is a viral expression vector, optionally a retroviral vector, a lentiviral vector, an adenoviral vector, an adeno-associated viral (AAV) vector, a virus-like particle (VLP), or a herpes simplex virus (HSV) vector.

In certain embodiments, the starting cell is a native cell or a differentiated cell. In certain embodiments, the starting cell is an engineered cell that is genetically engineered from a native cell or from a differentiated cell.

In certain embodiments, the differentiated cell is differentiated from a stem cell. In certain embodiments, the stem cell is a hematopoietic progenitor cell (*e.g.* T cell progenitor cell, NK cell progenitor cell, macrophage progenitor cell), hematopoietic stem cell (HSCs), CD34⁺ cell, a cell line of embryonic stem cell, mesenchymal stem cell or iPSC cell. The stem cell can be first modified to increase expression or activity of the ligand of the inhibitory checkpoint molecule, followed by gene editing in the endogenous inhibitory checkpoint molecule to reduce expression or activity of the endogenous inhibitory checkpoint molecule, the HLA class I protein and/or the HLA class II protein, and/or the endogenous TCR. After modification or engineering of the stem cells, the modified or engineered stem cells can be differentiated under suitable conditions to obtain the modified cells of the present disclosure. For differentiated hematopoietic lineage cells that genetically engineered to express recombinant TCR or CAR, the cells can be activated and expanded using methods as described, for example, in U.S. Patents 6,352,694. In certain embodiments, the starting cell and/or the modified cell can be expanded and/or activated *ex vivo* prior to transfusion.

The method provided herein can further comprise obtaining starting cells from a source, culturing cells, activating cells and expanding cells.

The method provided herein may also involve a step of isolating the starting cell (*e.g.*, immune cell) using various techniques known in the art, for example, flow cytometry. Briefly, a fluorescently labelled antibody with affinity to a marker of the starting cell (*e.g.*, immune cell) is used to label the starting cell in a sample. A gating strategy appropriate for the cells expressing the marker is used to segregate the cells. For example, T cells can be separated from other cells in a sample by using, for example, a fluorescently labeled antibody specific for a cell marker, such as CD4, CD8, CD28 and CD45 and corresponding gating strategy. In certain embodiments, T cells deficient for CD3 are separated or enriched.

Polynucleotides and Vectors

Suitable recombinant techniques can be used to construct the nucleic acids or polynucleotides provided herein encoding the ligand for the inhibitory checkpoint molecules, or encoding for the variant ligand, or encoding the PD-L1 variant, and the like. If needed, polynucleotide sequences encoding for one or more linkers are also operably linked to allow expression of the desired product.

The encoding polynucleotide sequence(s) can be mRNA, which can be *in vitro* transcribed, and optionally can be encapsulated in a suitable delivery vehicle such as a lipid nanoparticle.

The encoding polynucleotide sequence(s) can also be inserted into a vector for further cloning (amplification of the DNA) or for expression, using recombinant techniques known in the art. Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter (*e.g.* prokaryotic promoters such as T7, T7lac, Sp6, araBAD, trp, lac, tac, pLm, A3, lac, lpp, npr, pac, syn, trc and T3, or eukaryotic promoters such as SV40, CMV, and EF-1 α), and a transcription termination sequence.

In another aspect, the present disclosure provides an expression vector comprising the polynucleotide provided herein. In certain embodiments, the expression vector provided herein comprises sufficient cis-acting elements for expression, with other elements for expression to be provided by the host cell or in an *in vitro* expression system. In certain embodiments, the expression vector provided herein also comprises a nucleotide sequence encoding a signal peptide for, for example, nuclear localization, nucleolar localization, or mitochondrial localization.

In certain embodiments, the expression vector provided herein comprises the polynucleotide encoding a ligand of the present disclosure and polynucleotide(s) encoding one or more CARs or TCRs.

The expression vector provided herein can also comprise a polynucleotide encoding RNA-guided DNA endonuclease (*e.g.*, Cas9) and one or more nuclear localization sequences and optionally one or more deaminases.

The expression vector of the present disclosure can also include any suitable number of regulatory/control elements, *e.g.*, promoters, enhancers, introns, polyadenylation signals, Kozak consensus sequences, internal ribosome entry sites (IRES), or peptide 2A (P2A) sequence. These elements are well known in the art.

The expression vector can be selected from the group consisting of a viral vector, a virus-like particle (VLP), a plasmid, a minicircle, a nanoplasmid, a DNA vector, or an RNA vector. In certain embodiments, the expression vector is a viral expression vector, optionally a retroviral

vector, a lentiviral vector, an adenoviral vector an adeno-associated viral (AAV) vector, a virus-like particle (VLP), and a herpes simplex virus (HSV) vector. Generally, viral vectors comprise an origin of replication functional in at least one organism, a promoter, restriction endonuclease sites, and one or more selectable markers.

The expression vector provided herein can be a lentiviral vector, which is advantageous for long-term, stable integration of the polynucleotide encoding the CAR into the genome of non-proliferating cells that result in stable expression of the CAR in the host cell, *e.g.*, host T cell. Lentiviruses are complex retroviruses that are capable of infecting and expressing their genes in both mitotic and post-mitotic cells. The human immunodeficiency virus (HIV) is known as the most common lentivirus, which uses the envelope glycoproteins of other viruses to target a broad range of cell types. Preparation of lentiviruses has been described in Gustabo et al., Production and purification of lentiviral vectors. Nature Protocols volume 1, pages 241–245 (2006).

Other expression vectors, such as a plasmid vector, a phagemid vector, a phage derivative vector, a cosmid vector, a transposon vector, a site directed insertion vector (*e.g.*, CRISPR, Zinc finger nucleases, TALEN), an *in vitro* transcribed RNA vector, and a suicide expression vector are also within the contemplation of the present disclosure.

The expression vector provided herein can be delivered into a host cell using various techniques known in the art, for example, by physical, chemical or biological means. Physical methods for introducing an expression vector into a host cell include, without limitation, lipofection, calcium phosphate precipitation, particle bombardment, microinjection and electroporation. Biological methods include, without limitation, the use of viral vectors (especially retroviral vectors, *e.g.*, lentiviral vector) for inserting genes into host cells. Chemical means include, without limitation, colloidal dispersion systems (*e.g.*, macromolecule complexes, nanocapsules, microspheres, beads), and lipid-based systems (*e.g.*, oil-in-water emulsions, micelles, mixed micelles, and liposomes).

Pharmaceutical composition

In another aspect, the present disclosure also provides a pharmaceutical composition comprising the modified cells (*e.g.*, modified immune cells) provided herein, the recombinant cells provided herein, the proteins provided herein or the polynucleotides provided herein and a pharmaceutically acceptable medium. As used herein, the term “pharmaceutical composition” refers to a composition formulated for pharmaceutical use.

The term “pharmaceutically acceptable” indicates that the designated carrier, vehicle, diluent, excipient(s), and/or salt is generally chemically and/or physically compatible with the other ingredients comprising the formulation, and physiologically compatible with the recipient

thereof.

A “pharmaceutically acceptable medium” refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is bioactivity acceptable and nontoxic to a subject. Pharmaceutical acceptable medium for use in the pharmaceutical compositions disclosed herein may include, for example, pharmaceutically acceptable liquid, gel, or solid carriers, aqueous or nonaqueous vehicles, antimicrobial agents, buffers, antioxidants, isotonic agents, suspending/dispersing agents, sequestering or chelating agents, diluents, adjuvants, excipients, or non-toxic auxiliary substances, or various combinations thereof.

The pharmaceutical compositions of the present disclosure can be prepared using various techniques known in the art, see, for example, Remington, The Science And Practice of Pharmacy (21st ed. 2005). Briefly, the modified cell/ recombinant cell or population thereof is admixed with a suitable medium prior to use or storage. Suitable pharmaceutically acceptable medium generally comprise inert substances that help in: 1) administering the pharmaceutical composition to a subject, 2) processing the pharmaceutical compositions into deliverable preparations, and/or 3) storing the pharmaceutical composition prior to administration. In certain embodiments, the pharmaceutically acceptable medium comprises agents that can stabilize, optimize or alter the form, consistency, viscosity, pH, pharmacokinetics, and/or solubility of the formulation. Such agents include, without limitation, buffering agents, wetting agents, emulsifying agents, diluents, encapsulating agents, and skin penetration enhancers, for example, saline, buffered saline, dextrose, arginine, sucrose, water, glycerol, ethanol, sorbitol, dextran, sodium carboxymethyl cellulose, and combinations thereof.

Exemplary pharmaceutically acceptable medium include sugars (*e.g.*, lactose, glucose and sucrose), starches (*e.g.*, corn starch and potato starch), cellulose and derivatives thereof (*e.g.*, sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate), powdered tragacanth, malt, gelatin, lubricating agents (*e.g.*, magnesium stearate, sodium lauryl sulfate and talc), excipients (*e.g.*, cocoa butter and suppository waxes), oils (*e.g.*, peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil, glycols (*e.g.*, propylene glycol), polyols (*e.g.*, glycerin, sorbitol, mannitol and polyethylene glycol (PEG)), esters (*e.g.*, ethyl oleate and ethyl laurate), agar, buffering agents (*e.g.*, magnesium hydroxide and aluminum hydroxide), alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, pH buffered solutions, polyesters, polycarbonates, polyanhydrides, bulking agents (*e.g.*, polypeptides and amino acids, serum alcohols (*e.g.*, ethanol), (sterile) phosphate-buffered saline, Ringer's solution, dextrose solution and other non-toxic compatible substances used in pharmaceutical formulations).

Pharmaceutical compositions of the present disclosure may comprise the modified cell as

described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (*e.g.*, aluminum hydroxide); and preservatives. Compositions of the present disclosure are preferably formulated for intravenous administration.

Kit

In another aspect, the present disclosure also provides a kit comprising the modified cells (*e.g.*, modified immune cells) provided herein or the recombinant cells provided herein with reduced self-suppression, improved effector cell function, and improved cell activation and/or expansion. In another aspect, the present disclosure also provides a kit comprising the proteins provided herein, the polynucleotides or expression vectors provided herein for use in generating CAR-expressing or TCR-expressing modified cells (*e.g.*, modified immune cells) or recombinant cells with reduced self-suppression, improved effector cell function, and improved cell activation and/or expansion.

In some embodiments, the kits of the present disclosure comprise written instructions for the use of the kit. In certain embodiments, the instructions include at least one of the following: clinical studies, precautions, warnings, and/or references. The instructions can be either printed directly on the container (when present) or provided in the container or with the container as a label applied to the container, or as a separate sheet, pamphlet, card, or folder. Suitable containers include, for example, bottles, syringes, vials, and test tubes. The containers can be formed from a variety of materials such as plastic or glass. In certain embodiments, the container holds the pharmaceutical composition provided herein and have a sterile access port.

In certain embodiments, the kit further comprises a second container comprising a pharmaceutically acceptable medium as described above. In certain embodiments, the kit further comprises other materials that are commercially desirable or user friendly, such as other diluents, buffers, needles, filters, syringes, and package inserts with instructions for use.

Method of Treatment

In another aspect, the present disclosure provides a method of treating a condition or a disease in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of the modified cells (*e.g.*, modified immune cells) provided herein.

In certain embodiments, the subject has a disease selected from a cancer, an autoimmune disease, an infectious disease, aging, metabolic disease, and cardiovascular disease. Cell therapy have

been approved to treat cancer, and have been found to be useful in treating many other different conditions and diseases. More details have been reviewed in publications such as, Chu E. T. et al., *Cells*. 2020 Mar; 9(3): 563; Rurik J.G. et al, CAR T cells produced *in vivo* to treat cardiac injury, *Science* 375, 91–96 (2022); Aghajanian H. et al., Targeting cardiac fibrosis with engineered T cells, *Nature*, 573, 430-433(2019); Amor C. et al., Senolytic CAR T cells reverse senescence-associated pathologies, *Nature*, 583, 127-132 (2020); Wagner V. et al, T cells engineered to target senescence, *Nature*, 583, 37-38 (2020), Nezhad, M. S et al, Chimeric Antigen Receptor Based Therapy as a Potential Approach in Autoimmune Diseases: How Close Are We to the Treatment? *Front. Immunol.*, 26 November 2020, Stewart et al., Infectious complications of CAR T-cell therapy: a clinical update, *Ther Adv Infect Dis*. 2021 Jan-Dec; 8: 20499361211036773, which are incorporated herein to entirety.

In certain embodiments, the cancer is a hematological cancer or a solid tumor.

In certain embodiments, the condition or disease is a hematological cancer. In certain embodiments, the hematological cancer is a B cell cancer. In certain embodiments, the hematological cancer is selected from the group consisting of leukemia, myeloma, lymphoma, and combination thereof. In certain embodiments, hematological cancer is selected from the group consisting of T- cell acute lymphoblastic leukemia, mycosis fungoides, Sézary syndrome, Peripheral T lymphoma, NK/T-cell lymphoma, anaplastic large cell lymphoma ALK+, primary cutaneous T-cell lymphoma (CTCL), T-cell large granular lymphocytic leukemia (T-LGLL), angioimmunoblastic T/NK-cell lymphoma, Hepatosplenic T-cell lymphoma, primary cutaneous CD30 + lymphoproliferative disorders, extranodal NK/T-cell lymphoma (ENKTL), adult T-cell leukemia/lymphoma (ATLL), T-cell prolymphocytic leukemia (T-PLL), subcutaneous panniculitis-like T-cell lymphoma (SPTCL), primary cutaneous gamma-delta T-cell lymphoma (PCGD-TCL), aggressive NK-cell leukemia (ANKL), and enteropathy-associated T-cell lymphoma (EATL).

In certain embodiments, the condition or disease is a solid tumor. In certain embodiments, the solid tumor is selected from the group consisting of breast carcinoma, lung carcinoma, colorectal carcinoma, pancreatic carcinoma, glioma and lymphoma, head and neck tumors, neuroendocrine tumors, colorectal tumors, prostate tumors, breast tumors, lung tumors, such as small cell and non-small cell lung tumors, pancreatic tumors, thyroid tumors, ovarian tumors, cervical tumors, kidney tumors, brain tumors, liver tumors, Kaposi's sarcoma, CNS neoplasms, neuroblastomas, capillary hemangioblastomas, meningiomas, cerebral metastases, melanoma, gastrointestinal and renal carcinomas and sarcomas (*e.g.*, gastric cancer), rhabdomyosarcoma, glioblastoma, preferably glioblastoma multiforme, leiomyosarcoma, squamous cell carcinoma, basal cell carcinoma and skin cancers that can be treated by suppressing the growth of malignant keratinocytes, such as human malignant keratinocytes.

In certain embodiments, the autoimmune disease is selected from the group consisting of lupus erythematosus, rheumatoid arthritis and GVHD.

In certain embodiments, the autoimmune disease is a diffuse connective tissue disease.

In certain embodiments, the diffuse connective tissue disease comprises systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis (RA), idiopathic inflammatory myopathy (IIM), Churg-Strauss Syndrome and microscopic polyangitis.

In certain embodiments, the idiopathic inflammatory myopathy(IIM) includes polymyositis (PM), dermatomyositis (DM), amyopathic dermatomyositis (ADM), immune necrotizing myopathy (IMNM), inclusion body myositis (IBM) and juvenile dermatomyositis (JDM), but not limited to.

In certain embodiments, the lymphatic malignancies comprises B cell lymphoma and B lymphocyte leukemia. B cell lymphoma comprises Hodgkin's lymphoma(HL) and non-Hodgkin's lymphoma(NHL), and B lymphocyte leukemia comprises acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

In certain embodiments, infectious disease is selected from the group consisting of fungus infection, parasite/protozoan infection, malaria, coccidioiodmycosis immitis, histoplasmosis, onychomycosis, aspergilosis, blastomycosis, candidiasis albicans, paracoccidioiomycosis, microsporidiosis, Acanthamoeba keratitis, Amoebiasis, Ascariasis, Babesiosis, Balantidiasis, Baylisascariasis, Chagas disease, Clonorchiasis, Cochliomyia, Cryptosporidiosis, Diphyllbothriasis, Dracunculiasis, Echinococcosis, Elephantiasis, Enterobiasis, Fascioliasis, Fasciolopsiasis, Filariasis, Giardiasis, Gnathostomiasis, Hymenolepiasis, Isosporiasis, Katayama fever, Leishmaniasis, Lyme disease, Metagonimiasis, Myiasis, Onchocerciasis, Pediculosis, SARS-CoV infection, SARS-CoV-2 infection, Scabies, Schistosomiasis, Sleeping sickness, Strongyloidiasis, Taeniasis, Toxocariasis, Toxoplasmosis, Trichinosis, Trichuriasis, Trypanosomiasis, helminth infection, infection of hepatitis B (HBV), hepatitis C (HCV), herpes virus, Epstein-Barr virus, HIV-1, HIV-2, cytomegalovirus, herpes simplex virus type I, herpes simplex virus type II, human papilloma virus, adenovirus, Kaposi West sarcoma associated herpes virus epidemics, thin ring virus (Torquetenovirus), human T lymphotropic viruse I, human T lymphotropic viruse II, varicella zoster, JC virus and BK virus.

In certain embodiments, the recipient has or has a propensity to develop graft versus host disease (GVHD).

In certain embodiments, the recipient has or has a propensity to develop a neoplasia (e.g., leukemia).

In certain embodiments, the methods provided herein further comprise administering to the subject with one or more additional therapeutic agents, such as, one or more additional modified immune cells or a population thereof, immune checkpoint inhibitors (*e.g.*, anti-PD-L1 antibodies, anti-PD-1 antibodies), one or more cytokines (*e.g.*, IL-2, IFN- α , IFN- γ , or a combination thereof), or one or more chemotherapeutic agents (*e.g.*, cyclophosphamide, doxorubicin, vincristine, prednisone, rituximab, obinutuzumab, bendamustine, chlorambucil, cyclophosphamide, ibrutinib, methotrexate, cytarabine, dexamethasone, cisplatin, bortezomib, fludarabine, idelalisib, acalabrutinib, lenalidomide, venetoclax, cyclophosphamide, ifosfamide, etoposide, pentostatin, melphalan, carfilzomib, ixazomib, panobinostat, daratumumab, elotuzumab, thalidomide, lenalidomide, or pomalidomide, or a combination thereof). The one or more additional therapeutic agents as described above can be administered prior to, simultaneously, or after the administration of recombinant cell or the modified cells provided herein.

In another aspect, the present disclosure also provides a method of reducing recipient immune response to a transplant of cells, comprising administering to a subject a therapeutically effective amount of a transplant of the recombinant cell or the modified cells provided herein, thereby reducing the recipient immune response.

In another aspect, the present disclosure also provides method of transplanting cells to a subject with reduced risk of immune rejection, comprising administering to the subject a therapeutically effective amount of a transplant of the recombinant cell provided herein, or modified cells provided herein, wherein the recombinant cell or modified cells are allogeneic to the subject.

In some embodiments, a modified cell or recombinant cell of the present disclosure administered to the recipient proliferates *in vivo* and can persist in the subject for an extended period of time. When the modified cell is a modified immune cell, the modified immune cell can mature into memory immune cells and remain in circulation within the recipient and then generate a population of cells capable of actively responding to recurrence of a diseased or abnormal cells expressing the marker recognized by the CARs of the modified immune cell.

In certain embodiment, the recipient is administered with at least 1×10^4 cells, at least 5×10^4 cells, at least 1×10^5 cells, at least 5×10^5 cells, at least 1×10^6 cells, at least 5×10^6 cells, at least 1×10^7 cells, at least 5×10^7 cells, at least 1×10^8 cells, at least 5×10^8 cells, at least 1×10^9 cells, at least 2×10^9 cells, at least 3×10^9 cells, at least 4×10^9 cells, at least 5×10^9 cells, or at least 1×10^{10} cells. In certain embodiment, the recipient is administered at least 1×10^3 cells/kg of bodyweight, at least 5×10^3 cells/kg of bodyweight, at least 1×10^4 cells/kg of bodyweight, at least 5×10^4 cells/kg of bodyweight, at least 1×10^5 cells/kg of bodyweight, at least 5×10^5 cells/kg of bodyweight, at least 1×10^6 cells/kg of bodyweight, at least 5×10^6 cells/kg of bodyweight, at least 1×10^7 cells/kg of

bodyweight, at least 5×10^7 cells/kg of bodyweight, at least 1×10^8 cells/kg of bodyweight, at least 2×10^8 cells/kg of bodyweight, at least 3×10^8 cells/kg of bodyweight, at least 4×10^8 cells/kg of bodyweight, at least 5×10^8 cells/kg of bodyweight, or at least 6×10^8 cells/kg of bodyweight.

A person skilled in the art would understand that dosage of the pharmaceutical compositions provided herein may be determined based on various factors of the recipient, such as size, age, sex, weight, and condition. Dosages can be readily determined by a person skilled in the art from this disclosure and the knowledge in the art.

The person skilled in the art can readily determine the number of the modified/recombinant cells and the amount of optional additives, vehicles, medium and/or carriers in compositions and to be administered in methods of the present disclosure. Typically, additives, if any, are present in an amount of 0.001 to 50% (weight) solution in phosphate buffered saline, and the active ingredient (*e.g.*, the modified/recombinant cells provided herein) is present in the order of micrograms to milligrams, such as about 0.0001 to about 5 wt%, preferably about 0.0001 to about 1 wt%, still more preferably about 0.0001 to about 0.05 wt% or about 0.001 to about 20 wt%, preferably about 0.01 to about 10 wt%, and still more preferably about 0.05 to about 5 wt %.

It would be preferred to determine the toxicity of a certain dosage, such as by determining the lethal dose (LD) and LD50 in a suitable animal model (*e.g.*, a mouse). It would also be preferred to determine the timing of administering the composition(s), which elicit a suitable response. Such determinations do not require undue experimentation from the knowledge of the person skilled in the art and the present disclosure.

Administration of the pharmaceutical compositions provided herein can be performed using various conventional techniques including, without limitation, infusion, transfusion, or parenteral administration. In some embodiments, the parenteral administration includes infusing or injecting intravascularly, intratumorally, intravenously, intradermally, intramuscularly, intraarterially, transtracheally, intrathecally, intraperitoneally, subcutaneously, subcuticularly, intraarticularly, subcapsularly, subarachnoidly and intrasternally.

In certain embodiments, the pharmaceutical composition provided herein is administered locally to a diseased site (*e.g.*, tumor site). In certain embodiments, the pharmaceutical composition provided herein is administered to a subject by injection, by means of a catheter, by means of a suppository, or by means of an implant (*e.g.*, of a porous, non-porous, or gelatinous material, *e.g.*, a membrane, such as a sialastic membrane). In certain embodiments, the pharmaceutical composition provided herein is delivered in a controlled release system.

The method treatment provided herein results in an increased survival time of the recipient (*e.g.*,

human subject) as compared to the expected survival time of the recipient (*e.g.*, human subject) if the human subject was not treated with the pharmaceutical compositions provided herein. In another embodiment, the increase in the survival time of the human subject is at least 30 days. The increase in the survival time of the human subject can be at least 3 months, at least 6 months or at least 1 year. In certain embodiments, the recipient (*e.g.*, human subject) is intolerant and/or refractory to one or more other treatment of the disease and/or condition (*e.g.*, cancer). In certain embodiments, the recipient (*e.g.*, human subject) has had at least one unsuccessful treatment and/or therapy of the disease and/or condition (*e.g.*, cancer) prior to the method of treatment of the present disclosure. In certain embodiments, the recipient (*e.g.*, human subject) to be treated with the methods provided herein is a child (*e.g.*, 0-18 years of age) or an adult (*e.g.*, 18+ years of age).

In certain embodiments, the treatment methods of the present disclosure relate to using modified immune cells (*e.g.*, modified T cell) that express a CAR. The CAR can be specifically directed towards an antigen target, which is presented on unwanted cells (*e.g.*, cancer cell) in a host. In certain embodiments, the modified immune cells (*e.g.*, modified T cell) has enhanced cytotoxic response against its target. In certain embodiments, the modified immune cells (*e.g.*, modified T cell) induces an enhanced cytotoxic response against its target as compared to a reference cell (*e.g.*, native T cell).

In certain embodiments, the modified immune cells (*e.g.*, modified T cell) exhibits an enhanced cytotoxic response by at least 1.2-fold, 1.4-fold, 1.6-fold, 2-fold, 2.5-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 12-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 21-fold, 22-fold, 23-fold, 24-fold, 25-fold, 26-fold or more compared to a reference cell (*e.g.*, native T cell).

In some embodiments, the modified immune cells (*e.g.*, modified T cell) can kill at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 60%, at least 80%, at least 100%, at least 120%, at least 200%, at least 500%, at least 1000% or at least 2000% more target cells than a reference cell (*e.g.*, native T cell). In some embodiments, the modified immune cells (*e.g.*, modified T cell) is administered to an allogeneic host, wherein the modified immune cells (*e.g.*, modified T cell) has reduced or no (or negligible or minimum) rejection by the host.

EXAMPLES

The following examples further illustrate the present disclosure based on the drawings in detail, but the present disclosure is not limited thereto.

While the disclosure has been particularly shown and described with reference to specific

embodiments (some of which are preferred embodiments), it should be understood by those having skill in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the present disclosure as disclosed herein. It is to be understood that the foregoing description of two preferred embodiments is intended to be purely illustrative of the principles of the disclosure, rather than exhaustive thereof, and that changes and variations will be apparent to those skilled in the art, and that the present disclosure is not intended to be limited other than expressly set forth in the following claims.

Example 1: sgRNA design

The sgRNA sequences targeting HLA-A, HLA-B, TRAC, CIITA, and PD-1, respectively, were designed based on the genomic sequences, using the GPP sgRNA Designer (an online design tool at the Broad Institute, USA) on a whole-exon scale (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design>). Tools including "CRISPRko", "SpyoCas9 NGG", and "Human GRCh38" were selected to generate all potential sgRNA for these genes (Table 1 to Table 4), and the top 10 sgRNA of each gene were selected as the preferred sequences according to the priority.

According to the "NGG" characteristics in the target gene sequences, multiple sets of sgRNA sequences were designed to target the target DNA sequences in the upstream and downstream sequences of HLA-A and HLA-B genes (Table 1), CIITA (Table 2), and TRAC (Table 3), respectively. Table 1 below lists the target DNA sequences upstream of HLA-A (named with "AU"), downstream of HLA-A (named with "AD"), upstream of HLA-B (named with "BU"), and downstream of HLA-B (named with "BD") targeted by the sgRNAs.

Table 1 Target genes (HLA-A and HLA-B) and target DNA sequences

Target Genes	Target DNA Sequences(5'-3')
HLA-A	ctaaagaacataagttcacg (SEQ ID NO: 29)
HLA-A	atatgggctttagaataggg (SEQ ID NO: 30)
HLA-A	tctttattaatgtagccaca (SEQ ID NO: 31)
HLA-A	acagggcaaatgctgttttg (SEQ ID NO: 32)
HLA-A	acaaaacagcatttgccctg (SEQ ID NO: 33)
HLA-B	tctagatatccaatacccaa (SEQ ID NO: 34)
HLA-B	ctagatatccaatacccaat (SEQ ID NO: 35)
HLA-B	tatccaataccaatggggag (SEQ ID NO: 36)
HLA-B	atccaataccaatggggagt (SEQ ID NO: 37)
HLA-B	ggactttagaactgggaccg (SEQ ID NO: 38)

HLA-B	tgcgtggggactttagaact (SEQ ID NO: 79)
HLA-B	gaagccaatcagcgtcgccg (SEQ ID NO: 39)
HLA-B	aaagtccccacgcaccacc (SEQ ID NO: 80)
HLA-B	aggggggttgctgtcgaaaga (SEQ ID NO: 40)
HLA-B	gttgctgtcgaaagagggat (SEQ ID NO: 41)
HLA-B	tgaaattggacccgtcatca (SEQ ID NO: 42)
HLA-B	gaggggggttgctgtcgaaag (SEQ ID NO: 43)
HLA-B	gaccctcacactgagatag (SEQ ID NO: 44)
HLA-B	ttgctgtcgaaagagggatg (SEQ ID NO: 45)
HLA-A, HLA-B	cgcactcctggtcctgctcg (SEQ ID NO: 73)
HLA-A, HLA-B	ctatgccaacaacttgggc (SEQ ID NO: 74)
HLA-A, HLA-B	caactccttttaggcaacg (SEQ ID NO: 75)
HLA-A, HLA-B	tacacctccggtgttccc (SEQ ID NO: 76)
HLA-A, HLA-B	gctattcaactgccctgat (SEQ ID NO: 77)
HLA-A, HLA-B	agtttgaatagcacattggt (SEQ ID NO: 78)
HLA-A, HLA-B	tgcgtggggactttagaact (SEQ ID NO: 79)
HLA-A, HLA-B	aaagtccccacgcaccacc (SEQ ID NO: 80)
HLA-A, HLA-B	acctcatggagtgggagcct (SEQ ID NO: 81)
HLA-A, HLA-B	ctcatggagtgggagcctgg (SEQ ID NO: 82)
HLA-A, HLA-B	tacctcatggagtgggagcc (SEQ ID NO: 83)
HLA-A, HLA-B	gagcgaggggaccgcaggcg (SEQ ID NO: 84)
HLA-A, HLA-B	agcgaggggaccgcaggcgg (SEQ ID NO: 85)
HLA-A, HLA-B	aggagcgaggggaccgcagg (SEQ ID NO: 86)
HLA-A, HLA-B	ggagcgaggggaccgcaggc (SEQ ID NO: 87)
HLA-A, HLA-B	gggaggagcgaggggaccgc (SEQ ID NO: 88)
HLA-A	gagtccccgtgggtgcgtgcggg (SEQ ID NO: 89)
HLA-A	caggatgaaggaccctacgtagg (SEQ ID NO: 90)
HLA-A	acgacactgattggttctctgg (SEQ ID NO: 91)
HLA-A	gcgaccgcgacgacactgattgg (SEQ ID NO: 92)

HLA-A	ctacgtagggtccttcacctctgg (SEQ ID NO: 93)
HLA-A	tgaaggaccctacgtagggtggg (SEQ ID NO: 94)
HLA-A	cccagatcaagcttactctctgg (SEQ ID NO: 95)
HLA-A	gcactcaggcacaacttttccgg (SEQ ID NO: 96)
HLA-A	gaccctacgtagggtgggagagg (SEQ ID NO: 97)
HLA-A	agttcaggacagagataatggg (SEQ ID NO: 98)
HLA-A	cattatctctgtccctgaactgg (SEQ ID NO: 99)
HLA-A	accctacgtagggtgggagaggg (SEQ ID NO: 100)
HLA-A	atgaaggaccctacgtagggtgg (SEQ ID NO: 101)
HLA-A	gcgttcgcacaggagcagaggg (SEQ ID NO: 102)
HLA-A	cgttcgcacaggagcagagggg (SEQ ID NO: 103)
HLA-A	tccctctcccaacctacgtaggg (SEQ ID NO: 104)
HLA-A	gccagagagtaagcttgatctgg (SEQ ID NO: 105)
HLA-A	gagtcctgttgtaatgcttttgg (SEQ ID NO: 106)
HLA-A	gcggagttggggaatccccaagg (SEQ ID NO: 107)
HLA-A	catcctggatactcacgacgcg (SEQ ID NO: 108)
HLA-A	gaccaaccggggggatttttgg (SEQ ID NO: 109)
HLA-A	ggccaaaaatcccccggttgg (SEQ ID NO: 110)
HLA-A	ggcccgctccgtgggggatgaggg (SEQ ID NO: 111)
HLA-A	tttaggccaaaaatcccccggg (SEQ ID NO: 112)
HLA-A	gcccgtccgtgggggatgagggg (SEQ ID NO: 113)
HLA-A	accctcatccccacggacggg (SEQ ID NO: 114)
HLA-A	tcaggaccctcatccccacgg (SEQ ID NO: 115)
HLA-A	tggcgacctggcccgctccgtgg (SEQ ID NO: 116)
HLA-A	aggcgctgggcctctcccgggg (SEQ ID NO: 117)
HLA-A	tctccggggcaagggtctcggg (SEQ ID NO: 118)

HLA-A	gggcgacctggccccgtccgtggg (SEQ ID NO: 119)
HLA-A	gtctcgggggtcccgcggcttcgg (SEQ ID NO: 120)
HLA-A	ttctcgggggtcccgcggcttcggg (SEQ ID NO: 121)
HLA-A	gacccctcatccccacggacgg (SEQ ID NO: 122)
HLA-A	ctcgggggtcccgcggcttcgggg (SEQ ID NO: 123)
HLA-A	cgcccagccccgaccaaccggg (SEQ ID NO: 124)
HLA-A	gcaagggtctcgggggtcccgcgg (SEQ ID NO: 125)
HLA-A	gcgacctggccccgtccgtggggg (SEQ ID NO: 126)
HLA-A	gaccccgagacccttgccccggg (SEQ ID NO: 127)
HLA-A	gactggggtgaccgcgggggtcgg (SEQ ID NO: 128)
HLA-B	ttctctgaaggcaccgcataggg (SEQ ID NO: 129)
HLA-B	tttctctgaaggcaccgcatagg (SEQ ID NO: 130)
HLA-B	tcagcgtcgggtgaagagcattgg (SEQ ID NO: 131)
HLA-B	gataaaatacgcacactgcttgg (SEQ ID NO: 132)
HLA-B	gcgtattttatctgaacacttgg (SEQ ID NO: 133)
HLA-B	cgagggccgcgtctgcaatgggg (SEQ ID NO: 134)
HLA-B	tccgagggccgcgtctgcaatgg (SEQ ID NO: 135)
HLA-B	aacaggcagtgttcagcgtcgg (SEQ ID NO: 136)
HLA-B	tgtgtagggaactgagcacggg (SEQ ID NO: 137)
HLA-B	caatggggaggcgcagcgttggg (SEQ ID NO: 138)
HLA-B	gtgtagggaactgagcacgggg (SEQ ID NO: 139)
HLA-B	ccgagggccgcgtctgcaatggg (SEQ ID NO: 140)
HLA-B	cagcgtcgggtgaagagcattggg (SEQ ID NO: 141)
HLA-B	tcctcttgactcttgtgtaggg (SEQ ID NO: 142)
HLA-B	tcctacacaagagtccaagagg (SEQ ID NO: 143)
HLA-B	ctcctcttgactcttgtgtagg (SEQ ID NO: 144)

HLA-B	ttcccttcacttctctctctgg (SEQ ID NO: 145)
HLA-B	gggccgcgtctgcaatggggagg (SEQ ID NO: 146)
HLA-B	gcgcctccccattgcagacgcgg (SEQ ID NO: 147)
HLA-B	tctctatgagaatccctatgcgg (SEQ ID NO: 148)
HLA-B	aggtcctgcgccccgcctgcgg (SEQ ID NO: 149)
HLA-B	agtgcgggtcgggagggaatgg (SEQ ID NO: 150)
HLA-B	gagcgaggggaccgcaggcgggg (SEQ ID NO: 151)
HLA-B	tgcggtcccctcgctctcccgg (SEQ ID NO: 152)
HLA-B	agcgaggggaccgcaggcggggg (SEQ ID NO: 153)
HLA-B	aggagcgaggggaccgcaggcgg (SEQ ID NO: 154)
HLA-B	gggaaatggcctctgcggggagg (SEQ ID NO: 155)
HLA-B	ggagcgaggggaccgcaggcggg (SEQ ID NO: 156)
HLA-B	aggacctgaggagccgcggggg (SEQ ID NO: 157)
HLA-B	aggcgggggcgaggacctgagg (SEQ ID NO: 158)
HLA-B	caggacctgaggagccgcgggg (SEQ ID NO: 159)
HLA-B	gggaggagcgaggggaccgcagg (SEQ ID NO: 160)
HLA-B	gcctctgccgggaggagcgaggg (SEQ ID NO: 161)
HLA-B	acctgaggagccgcggggagg (SEQ ID NO: 162)
HLA-B	ggagggaaatggcctctgccggg (SEQ ID NO: 163)
HLA-B	tgaggagccgcggggaggagg (SEQ ID NO: 164)
HLA-B	cctctgccgggaggagcgagggg (SEQ ID NO: 165)
HLA-B	ggcctctgccgggaggagcgagg (SEQ ID NO: 166)
HLA-B	gggagggaaatggcctctgccgg (SEQ ID NO: 167)
HLA-B	tcctccggcgcggtcctcagg (SEQ ID NO: 168)

Table 2 Target genes (CIITA) and target DNA sequences

Target Genes	Target DNA sequences(5'-3')
CIITA	CTGGTCCAGTTCCGCGATAT (SEQ ID NO: 46)
CIITA	TTATGCCAATATCGCGGAAC (SEQ ID NO: 47)
CIITA	GATATTGGCATAAGCCTCCC (SEQ ID NO: 48)
CIITA	ACTGGACCAGTATGTCTTCC (SEQ ID NO: 49)
CIITA	TCAACTGCGACCAGTTCAGC (SEQ ID NO: 50)
CIITA	GGAGGCTTATGCCAATATCG (SEQ ID NO: 51)
CIITA	TAGGGGCCCAACTCCATGG (SEQ ID NO: 52)
CIITA	CTGACAGGTAGGACCCAGCA (SEQ ID NO: 53)
CIITA	AGGCTGTTGTGTGACATGGA (SEQ ID NO: 54)
CIITA	AGGTGATGAAGAGACCAGGG (SEQ ID NO: 55)
CIITA	ATAGAAGTGGTAGAGGCACA (SEQ ID NO: 56)
CIITA	AGAAGTGGTAGAGGCACAGG (SEQ ID NO: 57)

Table 3 Targeted genes (TRAC) and target DNA sequences

Genes Targeted	Target DNA Sequence(5'-3')
TRAC	AGAGCAACAGTGCTGTGGCC (SEQ ID NO: 58)

Table 4 Targeted genes (PD-1) and target DNA sequences

Target Genes	Target DNA Sequences(5'-3')
PD-1	CGACTGGCCAGGGCGCCTGT (SEQ ID NO: 59)

Based on the target DNA sequences listed in Table 1 to Table 4 above, sgRNA sequences were designed and synthesized as follows. Each sgRNA in this application is composed of a 5' targeting fragment and a 3' Cas-binding fragment. The 5' targeting fragment is complementary to the target site in the genome, and is otherwise identical to the 20-nucleotide sequence (i.e. the target DNA sequence in Table 1 to Table 4 above) upstream of the PAM sequence NGG, except that all the thymine (T) is replaced by uracil (U). The 3' Cas-binding fragment is a consensus motif and has a nucleotide sequence as follows:

GUUUUAGAGCUAGAAAUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUG
AAAAAGUGGCACCGAGUCGGUGCUUUU (SEQ ID NO: 60).

Take sgRNA targeting PD-1 as an example, the 5' target DNA sequence has a sequence of CGACTGGCCAGGGCGCCTGT (SEQ ID NO: 59) except for substitution of T to U, which is linked at its 3' end to the consensus motif of SEQ ID NO: 59. The complete sgRNA sequence (5' -3 ') targeting PD-1 is shown below:

CGACUGGCCAGGGCGCCUGUGUUUUAGAGCUAGAAAUAGCAAGUAAAAUAAG
GCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU (SEQ ID
NO: 61).

It should be noticed that each sgRNA of this Example was chemically modified, and the specific modifications included phosphorothioate interlinkage and 2'-O-methyl (2-OMe modified) ribose sugar modification at the first 3 nucleotides at the 5' end as well as the 2nd, 3rd, and 4th nucleotides at the 3' end of the sgRNA. The final chemically modified sgRNA sequence is set forth below, in which the modified nucleotides are in bold and italic format, with an asterisk symbol (*):

C*G*A*CUGGCCAGGGCGCCUGUGUUUUAGAGCUAGAAAUAGCAAGUAAAAUA
AGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC***U*U*U****U
(SEQ ID NO: 62)

Unless otherwise specified, all sgRNA sequences converted from target DNA sequences (in Table 1-Table 4) are synthesized by the above rules, and sgRNAs are prepared by chemical synthesis.

Example 2: Construction of chimeric antigen receptor (CAR) expression vector

This Example illustrates construction of an expression vector, with a CAR expression cassette expressing a CAR linked to a truncated PD-L1 (SEQ ID NO: 19) by P2A (Figure 1A).

The CAR contains an anti-CD19 single-chain Fv (scFv) derived from antibody FMC63 (CD19-scFv), followed by the CD8 α transmembrane region (CD8 α TM), 4-1BB, and CD3 ζ .

The truncated PD-L1 (abbreviated as TrPD-L1 herein), compared with wild-type PD-L1 (1-290 aa, SEQ ID NO: 12), lacks the sequence from position 260 to 290 at the C-terminus.

To construct the CAR expression cassette, the DNA sequences encoding the following elements was synthesized and ligated in the order of 5' to 3': signal peptide (amino acid sequence SEQ ID NO: 21), light chain variable region of CD19 antibody FMC63 (amino acid sequence SEQ ID NO: 22), linker (amino acid sequence SEQ ID NO: 23), heavy chain variable region of CD19 antibody FMC63 (amino acid sequence SEQ ID NO: 24), hinge region (amino acid sequence SEQ ID NO: 25), CD8 α transmembrane domain (TM) (amino acid sequence SEQ ID NO: 26),

4-1BB costimulatory signal transduction region (amino acid sequence SEQ ID NO: 27), CD3 ζ signal transduction domain (amino acid sequence SEQ ID NO: 28), P2A (Amino acid sequence SEQ ID NO: 16) and the truncated PD-L1 (TrPD-L1) (amino acid sequence SEQ ID NO: 8). The resulting expression cassette encodes a fusion protein CD19 CAR-P2A-TrPD-L1 having a full-length amino acid sequence of SEQ ID NO: 19, and the polynucleotide sequence is shown in SEQ ID NO: 20).

A control CAR expression cassette was also constructed, by replacing the TrPD-L1 sequence in the CD19 CAR-P2A-TrPD-L1 expression cassette described above with the wild-type PD-L1 sequence, to create a control CD19 CAR-P2A-wtPD-L1 expression cassette.

The CD19 CAR-P2A-TrPD-L1 expression cassette or the CD19 CAR-P2A-wtPD-L1 control expression cassette described above was integrated between the 5- and 3-terminal LTR sequences, respectively, of pELPS lentiviral vector (purchased from Kingsley Biotechnology) (Figure 1B and Figure 6), to construct the CAR expression master plasmid CD19DL1-01 or control plasmid CD19wtL1, which was subsequently packaged into lentivirus according to the instructions provided by the supplier.

Example 3: Lentiviral packaging and detection

CD19DL1-01 plasmid, together with lentiviral packaging plasmids PMDLG/PRRE, PRSV/rev (purchased from Wuxi Shengji Medical Technology Co., Ltd.) and lentiviral envelope plasmid pMD2. G (Wuxi Shengji Medical Technology Co., Ltd.) were transfected into 293T cells, using the transfection reagent PEI (Polyetherimide, purchased from Polysciences, Inc). The transfected 293T cells were cultured and the culture supernatant was harvested and concentrated by ultrafiltration and ultracentrifugation to obtain lentivirus for expression of CD19 CAR-P2A-TrPD-L1.

The virus titer was detected by flow cytometry. Specifically, the lentivirus solution for expression of CD19 CAR-P2A-TrPD-L1 was diluted 100-fold, and added at 1 μ l/well, 10 μ l/well, 20 μ l/well, 30 μ l/well, or 50 μ l/well to 293T cells cultured in 24-well plates for 8 hours. After 24 hours, virus-infected 293T cells were centrifuged, resuspended and adjusted to a cell density of 1×10^6 /ml, followed by addition of biotin-CD19 antigen at a final concentration of 1 μ g/ml, and then incubation with the secondary antibody APC-Streptavidin (purchased from BD), before detection of the expression of CD19 CAR on the 293T cells by flow cytometry.

The experimental results (Figure 2) showed that the virus solution prepared by the virus packaging system of this example successfully infected 293T cells and had good infection ability. The CD19 CAR was also expressed in the virus-transfected 293T cells and was able to recognize the CD19 antigen. The calculation result shows that the virus titer was 2.79×10^8 TU/ml

(transduction unit/ml).

In a similar manner, lentiviral packaging was performed with the control plasmid CD19wtL1 prepared from Example 2 and the control virus for expression of CD19 CAR-P2A-wtPD-L1 was obtained and tested. The results showed that the control virus can also induce CD19 CAR expression in 293T cells (data not shown).

Example 4: T cell isolation and activation

PBMC cells were taken out from the liquid nitrogen tank, recovered and counted. The total amount of PBMC cells was calculated according to the cell count. The incubation system was prepared. CD4 and CD8 magnetic sorting beads were added for sorting. Finally, *ex-vivo* culture medium containing IL-2 and 10% FBS was added according to the total amount of T cells sorted. T cell TransAct magnetic beads were added. After mixing, they were placed in a 37°C, 5% CO₂ incubator to activate and culture for two days.

Example 5: Viral Transfection of Activated T cells

To prepare T cells expressing the CD19-CAR and the truncated PD-L1 TrPD-L1, the T cells were activated according to Example 4 (after 48 hours activation) and were adjusted to a density of 1×10^6 /ml, followed by transfection at a Multiplicity of Infection (MOI) of 15 with CD19 CAR-P2A-TrPD-L1 lentivirus obtained from Example 3. Cells were collected 24 hours after virus infection, and the expression of CD19-CAR and the truncated PD-L1 TrPD-L1 was detected by flow cytometry. The results showed that both CD19-CAR (see Figure 3A) and truncated PD-L1 TrPD-L1 (see Figure 3B) could be highly expressed by the infected T cells, and the proportion of the two was synchronous (the positive rates of expression of CD19-CAR and TrPD-L1 were 37.42% and 41.49%, respectively), indicating that the gene overexpression system used was efficient, stable, and consistent. The resulting T cells expressing CD19-CAR and the truncated PD-L1 TrPD-L1 are referred to as TrPDL1-CART cells in this application.

As a control, T cells activated for 48 hours were infected with CD19 CAR-P2A-wtPD-L1 lentivirus obtained from Example 3 using the same method, and T cells expressing CD19-CAR and full length wtPD-L1 obtained were referred to as PDL1-CART cells in this application.

Example 6: Preparation of universal CART for T cell electroporation and flow cytometry

In order to prepare for universal CAR-T cells (also known as UCART), target genes TRAC, HLA-A, HLA-B, CIITA and PD-1 were knocked out in the TrPDL1-CART cells or PDL1-CART cells prepared in Example 5.

TrPDL1-CART cells or PDL1-CART cells were collected and counted, gently mixed with electroporation buffer and RNP (Ribonucleoprotein, ribonucleoprotein complex, the main

component is the complex formed by Cas9 protein and sgRNAs as prepared in Example 1), before initiation of electroporation. After electroporation, the mixture was cultured in pre-warmed medium. After electroporation, the cells were sampled and counted every 2 days, and the key parameters such as cell survival rate, total viable cell count and cell diameter were recorded. After culturing for 7 days, the knockout efficiency of related genes was detected.

The obtained TrPDL1-CART cells knocked out of the above target genes (TRAC, HLA-A, HLA-B, CIITA and PD-1) were called TrPDL1-UCART cells. The obtained PDL1-CART cells knocked out of the above target genes (TRAC, HLA-A, HLA-B, CIITA and PD-1) are called PDL1-UCART cells.

On day 7 after electroporation, sufficient numbers of native T cells, TrPDL1-CART obtained from Example 5, and TrPDL1-UCART cells obtained from this Example were detected by flow cytometry for expression of HLA-A, HLA-B, HLA-DR, and CD3. The detection antibodies were diluted in dark using staining buffer according to the suggested dilution ratio shown in the following table.

Table 5 Antibody used in the present example

Antibody name	Cat. No.	Dilution ratio
AF647 Anti-HLA-A Ab	BD biosciences, 567732	1:100
HLA-B Ab	BD biosciences, 752626	1:50
PE HLA-DR Ab	BD biosciences, 560651	1:100
APC anti-human CD3 Ab	Biolegend, 300312	1:125

The results showed that the above genes had been efficiently knocked out in TrPDL1-UCART cells, with the positive rates of HLA-A, HLA-B, HLA-DR and CD3 expression decreased to 25.38%, 4.67%, 23.85% and 23.83%, respectively. The experimental results are shown as follows in Table 6.

Table 6. The positive rates of HLA-A, HLA-B, HLA-DR and CD3 expression in cells

Cell Name	SgRNA: cas9	CD3	HLA-A	HLA-B	HLA-DR
Native T cells	--	98.03%	97.18%	32.94%	58.69%
TrPDL1-CART	--	96.69%	98.00%	16.28%	48.61%
TrPDL1-UCART	1:1	23.83%	25.38%	4.67%	23.85%

Example 7: Enrichment of CD3 negative cells

In order to enrich CD3-negative cells, TrPDL1-UCART cells obtained from Example 6 were resuspended with an appropriate amount of enrichment buffer at a proper density, followed by addition of anti-human CD3 magnetic beads proportional to the total number of cells, and enrichment of CD3-negative cells by magnetic bead column chromatography. Appropriate amount of purified cells was taken for flow cytometry to detect the positive rate of CD3, and the remaining cells were placed in a 37°C, 5% CO₂ incubator overnight. Table 7 below shows enrichment results as detected by flow cytometry.

Table 7. Positive rate of CD3 expression in cells

Cell Name	Blank	CD3 +
Native T cell	0.00%	98.86%
CART	0.04%	98.85%
TrPDL1-UCART (before enrichment)	0.02%	25.60%
TrPDL1-UCART (after enrichment)	0.00%	0.36%

The results showed that the positive rate of CD3 was 25.6% before enrichment (i.e., 25.6% of TrPDL1-UCART cells expressed CD3), and decreased to 0.36% after enrichment (i.e., only 0.36% of enriched TrPDL1-UCART cells expressed CD3). This showed that the purity of CD3-negative TrPDL1-UCART cells was greatly improved.

Example 8: Detection of cytotoxicity of TrPDL1-CART and TrPDL1-UCART

To test the cytotoxicity of TrPDL1-UCART, cell killing experiments were performed using the enriched CD3-negative TrPDL1-UCART obtained in Example 7 as effector cells and luciferase-expressing Raji cells (B cells) as target cells. As a control, native T cells or TrPDL1-CART cells obtained from Example 5 were used as effector cells in parallel control experiments, using the same target cells.

Sufficient effector and target cells were obtained, and the effector cells were diluted to a density of $4.0 \times 10^6/\text{ml}$ and the target cells were diluted to a density of $2.0 \times 10^5/\text{ml}$. The TrPDL1-CART was determined to have anti-CD19 CAR expression rate of 63.05%, and TrPDL1-UCART was determined to have anti-CD19 CAR expression rate of 75.58%. To ensure the level of anti-CD19 CAR of these cells were comparable, the volume of TrPDL1-CART cells and TrPDL1-UCART cells were further adjusted such that to TrPDL1-UCART was diluted by 1:1.19.

Effector cells of TrPDL1-CART and TrPDL1-UCART were then further diluted with X-VIVO complete medium according to the following gradient 20:1, 15:1, 10:1, 5:1, 2:1, and 1:1.

The effector cells (native T cells, TrPDL1-CART or TrPDL1-UCART) were mixed with target cells at 50 μ L/well : 50 μ L/well. After 24 h and 48 h of culture, 100 μ L/well of the substrate of Luciferase was added, and the OD values were read by a microplate reader to detect Luciferase activity in the cells.

The results (Figure 4A and Figure 4B) showed that TrPDL1-CART and TrPDL1-UCART could significantly kill the target cells, and the killing effect was enhanced with the increase of effector:target (E:T) ratio, indicating that the CART and UCART cells expressing the truncated PD-L1 as constructed according to the present disclosure had good cytotoxicity on the target cells.

Example 9: ELISA detection of IFN- γ and TNF- α secreted by TrPDL1-CART and TrPDL1-UCART

To test the cytotoxicity, TrPDL1-UCART was used as effector cells and Raji cells (B cells) expressing luciferase were used as target cells, and the cytokine TNF- α and IFN- γ release was detected after 24 hours of incubation of effector cells with target cells. As a control, native T cells or TrPDL1-CART cells obtained from Example 5 were tested as effector cells in parallel control experiments.

The cell supernatant after 24 hours of co-culture was transferred into a new centrifuge tube, centrifuged to remove cell debris, and the supernatant was taken to detect TNF- α and IFN- γ by ELISA.

The results showed (Figure 5A and Figure 5B) that both TrPDL1-CART and TrPDL1-UCART could significantly release TNF- α and IFN- γ after co-culture with the target cells, indicating that the universal CAR-T cells constructed according to the present disclosure had normal cytokine expression ability.

Example 10: MLR (Mixed Lymphocyte Reaction) experiment with allogeneic PBMC

To evaluate whether CAR-T cells expressing the truncated PD-L1 TrPDL1 can have reduced immune rejection from an allogeneic host immune system *in vivo*, mixed lymphocyte reaction experiments were performed, where peripheral blood mononuclear cells (PBMCs) from allogeneic individuals were used as effector cells, and CAR-T cells expressing the truncated PD-L1 TrPDL1 were used as target cells.

Different target cells were tested, including: 1) natural T cells; 2) TrPDL1-B2M^{-/-}-CART cells; 3) TrPDL1-CART cells (prepared in Example 5); 4) TrPDL1-UCART cells (prepared in Example 6 and enriched for CD3 negativity in Example 7).

TrPDL1-B2M^{-/-}-CART cells were prepared using methods similar to that described in Example 6. Briefly, the TrPDL1-CART cells obtained from Example 5 were knocked out using sgRNA

targeting B2M, and the knockout efficiency was detected by a method similar to that shown in Example 6. The results showed that B2M expression rate was 2.25% in the TrPDL1-B2M^{-/-} -CART cells.

The mixed lymphocyte reaction between the above target cells and allogeneic PBMC was performed as follows.

Preparation of effector cells: allogeneic monocytes and PBMC cells were centrifuged at 1200 rpm and counted, washed with PBS and resuspended. The cell density was adjusted to $2 \times 10^5/\text{ml}$ for future use.

Preparation of target cells: Native T cells, TrPDL1-B2M^{-/-} -CART cells, TrPDL1-CART cells and TrPDL1-UCART cells were irradiated with 2 Gy to destroy their expansion ability, then stained with 5 μM cell proliferation dye CFSE for 15 minutes at room temperature, and the cell density was adjusted to $2 \times 10^5/\text{ml}$ for future use.

Mixed lymphocyte reaction: the stained target cells and allogeneic PBMC were co-cultured as a proper proportion in a 96-well culture plate, and the cells were collected after culture for 24h.

The proportion of CD45⁺ CFSE⁺ double-positive cells was measured by flow cytometry to calculate the efficiency of target cell removal in the mixed lymphocyte reaction. If allogeneic PBMC cells have immune rejection on the target cells, the target cells will lyse and the cell membrane will be disrupted, so the CFSE fluorescence on the target cell surface will not be detected. CD45 is a pan-leukocyte marker, which is used to localize leukocytes, and exclude non-leukocytes in PBMCs so as to make the localization of target cells more accurate.

The experimental results are shown in Table 8 below. The results showed that when TrPDL1-UCART was co-cultured with PBMCs derived from allogeneic individuals, it could significantly inhibit the immune rejection from the allogeneic PBMCs, and avoid being removed by allogeneic PBMCs. In contrast, TrPDL1-B2M^{-/-} -CART cells and TrPDL1-CART cells were still removed by allogeneic PBMCs. This suggests that TrPDL1-UCART can significantly reduce immune rejection against allogeneic cells and was significantly more effective than universal CAR-T cells with only B2M knockout.

Table 8

Target ratio	Residual proportion of CD45 + CFSE + double positive target cells			
	T	TrPDL1-B2M ^{-/-} -CART	TrPDL1-CART	TrPDL1-UCART
1:1	20.5%	31.33%	19.85%	76.56%
2:1	7.6%	17.58%	4.33%	74.08%
5:1	0.98%	1.01%	0.28%	81.38%

Example 11: Effects of PD-1 Engagement on TrPDL1-UCART and PDL1-UCART cells on B cells

Both PD-1 and PD-L1 can mediate inhibitory signaling to T cells. The full-length PD-L1 sequence contains an intracellular domain. To verify whether truncation of the intracellular domain of PD-L1 may reduce the inhibitory signaling transmitted to T cells upon PD-L1 activation, we compared the cell proliferation and cytotoxicity of the UCAR-T cells expressing full-length wtPD-L1 and UCAR-T cells expressing the truncated PD-L1 TrPDL1, in the presence of PD-1-expressing cells.

Preparation of effector cells: CD19-binding TrPDL1-UCART cells and PDL1-UCART cells were prepared according to Example 6, followed by staining with 5 μ M cell proliferation dye CFSE for 15 mins at room temperature, and was adjusted to a cell density of 2×10^5 /ml for future use.

Preparation of target cells: Raji-luciferase cells (B cells) expressing PD-1 were obtained by recombinantly expressing human PD-1 on the Raji-luciferase cells provided in Example 8. The Raji-luciferase cells also naturally express CD19. The cell density was adjusted to 2×10^5 /ml for future use.

Mixed lymphocyte reaction: The effector cells and target cells prepared above were mixed and cultured in a 96-well culture plate at an appropriate ratio, and the cells were collected after 0 h, 24 h, 48 h and 72 h, respectively.

The proliferation of effector cells and the efficiency of target cell removal in mixed lymphocyte reactions were evaluated by measuring the proportion of CFSE + positive cells and CD19 positive cells by flow cytometry.

The results are shown in Table 9 and Table 10 below. The results showed that in the presence of cells expressing PD-1, the PDL1-UCART cell expressing full-length PD-L1 suffered from significant inhibition in both proliferation and target cell cytotoxicity, with the proliferation at 72 hours was only 1 fold and the killing effect on target cells was only less than 40%. In contrast, UCART expressing the truncated PD-L1 TrPDL1 not only maintained high cell proliferation, with a fold proliferation of more than 17 at 72 hours, but also showed significant killing of target cells, with 99% killing of target cells observed at 72 hours. This suggests that truncated PD-L1 TrPDL1 can significantly reduce the PD-L1-mediated inhibition following PD-1 engagement, and can greatly improve both UCART cell proliferation and cell killing effect.

Table 9

Time	Fold Proliferation of CFSE Positive Effector Cells	
	PDL1-UCART	TrPDL1-UCART
0h	0	0
24h	0.64	1.12
48h	0.85	3.26
72h	1.01	17.80

Table 10

Time	Residual rate of CD19-positive cells	
	PDL1-UCART	TrPDL1-UCART
0h	98.1%	99.1%
24h	84%	20.4%
48h	75.3%	3.71%
72h	61.9%	0.05%

Example 12: Evaluation of UCART without PD-1 knockout

We further tested the proliferation and cytotoxicity of truncated PD-L1 expressed UCAR-T cells with or without PD-1 knockout.

Preparation of effector cells: TrPDL1-UCART was prepared according to the method shown in Example 6, in which PD-1 was knocked out and the truncated PD-L1 TrPDL1 was expressed. A variant cell of TrPDL1-UCART without PD-1 knockout, which expresses both PD-1 and the truncated PD-L1, was prepared using similar methods as described in Example 6, and was referred to as PD-1+ TrPDL1-UCART. Both TrPDL1-UCART and PD-1+ TrPDL1-UCART were stained with 5 μ M cell proliferation dye CFSE for 15 min at room temperature, and the cell density was adjusted to 2×10^5 /ml for future use.

Preparation of target cells: the cell density of Raji-luciferase cells (B cells) provided in Example 8 were adjusted to 2×10^5 /ml for future use. This cell also naturally expresses CD19.

Mixed lymphocyte reaction: The above effector cells and target cells were mixed in a 96-well culture plate at an appropriate ratio, and 100 μ l of target cells and effector cell suspension was added to each well, with 3 duplicate wells for each group. The cells were cultured at 37°C in a 5% CO₂ incubator, and the cells were collected after 0 h, 24 h, 48 h and 72 h.

The proportion of CFSE positive effector cells and CD19 positive target cells was measured by

flow cytometry to evaluate the proliferation of effector cells and the efficiency of target cell clearance in the mixed lymphocyte reaction. The results are shown in Tables 11 and 12 below.

Table 11

Time	Fold Proliferation of CFSE Positive Effector Cells	
	PD-1 + TrPDL1-UCART (PD-1 Expression)	TrPDL1-UCART (PD-1 knockout)
0h	0	0
24h	1.21	2.28
48h	2.03	5.83
72h	1.88	11.82

Table 12

Time	Residual rate of CD19 positive target cells	
	PD-1 + TrPDL1-UCART (PD-1 Expression)	TrPDL1-UCART (PD-1 knockout) -
0h	92.1%	90.55%
24h	79.08%	17.22%
48h	64.52%	2.10%
72h	47.83%	0.02%

The results showed that when UCART cells expressed both PD-1 and PD-L1, their cell proliferation and cytotoxicity were significantly inhibited, where the proliferation at 72 hours was 1.88 fold, and the cytotoxicity to target cells was only less than 50%. Since the target cells used in this experiment do not express PD-1, it is speculated that the interaction between the PD-1 expressed on one UCART cell and the PD-L1 expressed on another UCART cell, which leads to the self-inhibition of the proliferation and cytotoxicity of the UCART cells expressing both PD-1 and PD-L1.

In contrast, when UCART cells expressed PD-L1 was knockout of PD-1, they not only maintained high cell proliferation, and the proliferation at 72 hours was more than 11 fold, but also showed significant increase in cytotoxicity, with 99% killing effect on target cells at 72 hours. This suggests that knockout of the endogenous inhibitory checkpoint molecule while expressing the ligand for the knocked-out checkpoint on the UCART cells can significantly improve the proliferation and cytotoxicity of UCART cells and avoid self-inhibition between the UCART cells.

The following examples relate to treatments of scleroderma, immune necrotizing myopathy (IMNM) and Systemic Lupus Erythematosus (SLE), respectively. The baseline information of candidate subjects enrolled in the following studies is shown in Table 13-1 and Table 13-2.

A total of 13 subjects were enrolled, including 6 with refractory scleroderma, 5 with refractory SLE, and 2 with refractory IMNM. Among these subjects, 4 were males and 9 were females. The subjects had a mean age of 41 years (range: 18-63 years), a mean weight of 59.3 kg (range: 35.0-92.0 kg), and a median disease duration of 7 years (range: 1-20 years). All subjects received at least one immunosuppressive therapy with no or poor response, including glucocorticoids, hydroxychloroquine, mycophenolate mofetil, tacrolimus, cyclophosphamide, azathioprine, tocilizumab, methotrexate, telitacicept, belimumab, and rituximab. The baseline characteristics are summarized in Table 13.

Table 13-1 Baseline of subjects

	IMNM-01	IMNM-02	SLE-01	SLE-02	SLE-03	SLE-04	SLE-05
Age (years)	42	22	18	37	54	33	25
Gender	Female	Male	Female	Female	Female	Female	Female
Disease	IMNM	IMNM	SLE	SLE	SLE	SLE	SLE
Disease duration (years)	10	15	9	11	20	12	6
Organ involvement							
Number	3	2	2	3	2	3	2
Skin	+		+	+	+	+	+
Joint	+			+		+	
Muscle	+	+					
Lung							
Kidney		+	+	+	+	+	+
Digestive system							
Cardiovascular system							
Prior lines of therapy	7	7	5	5	9	5	5
Total dose ($\times 10^6$ cells)	58.65	49.34	91.26	56.58	59.14	64.50	49.34
Note: IMNM: Immune-mediated necrotizing myopathy; SLE: Systemic lupus erythematosus							

Table 13-2 Baseline of subjects

	Sd-01	Sd-02	Sd-03	Sd-04	Sd-05	Sd-06
Age (years)	45	56	25	63	57	60

Gender	Male	Male	Female	Male	Female	Female
Disease	Sd	Sd	Sd	Sd	Sd	Sd
Disease duration (years)	3	1	5	1	4	7
Organ involvement						
Number	5	3	5	2	4	5
Skin	+	+	+		+	+
Joint	+	+	+	+		+
Muscle						
Lung	+	+	+	+	+	+
Kidney						
Digestive system	+		+		+	+
Cardiovascular system	+		+		+	+
Prior lines of therapy	6	5	4	4	5	5
Total dose ($\times 10^6$ cells)	58.65	58.65	36.50	58.00	67.20	56.00
Note: Sd: Scleroderma						

The method of lymphodepletion pretreatment and administration is as follows:

Lymphodepletion pretreatment

Assessment was performed in candidate subjects before lymphodepletion pretreatment. Qualified subjects were enrolled on Day 5 before first injection with UCART cells prepared according to the method shown in Example 6.

The scheme for lymphodepletion is as follows:

Fludarabine: 25 mg/m², i.v. drip for 3 days on Day 3, Day4, and Day5;

Cyclophosphamide: 300 mg/m², i.v. drip for 2 days on Day4, and Day5.

Or, the dosages of fludarabine and cyclophosphamide in the scheme could be adjusted according to the absolute value of lymphocytes in peripheral blood.

Evaluation for treatment with UCART was performed after completion of lymphodepletion pretreatment.

Treatment with UCART

Subjects who passed the evaluation were subject to intravenous infusion of UCART once on D0,

and hospitalized for observation for at least 7 days. All subjects received UCART at the dose of 1.0×10^6 cells/kg after lymphodepletion with fludarabine $25 \text{ mg/m}^2 \times 3$ days and cyclophosphamide $300 \text{ mg/m}^2 \times 2$ days, with an average total infusion dose of 59.34×10^6 cells (range: 36.50×10^6 - 91.26×10^6 cells). DLT observation period is started from the day of infusion to the 30th day after infusion.

Example 13: Evaluation of UCART treatment in Scleroderma

Therapeutic Response Evaluation

As is shown in Figure 8A and Figure 8B, the numbers of B cells decreased and UCART cells were increased remarkably, as well as CAR copies. Meanwhile, the secretion level of cytokine IL-6 was low during the process of treatment.

Results

All subjects achieved a Composite Response Index in Systemic Sclerosis (CRISS) score of over 0.6 two months after administration, with 83.3% (5/6) of them reaching 0.99, indicating significant improvement in all subjects. 83.3% (5/6) subjects exhibited improvements in Forced Vital Capacity (FVC) percentage compared to baseline. All subjects (100%, 6/6) had significant decreases in modified Rodnan skin (mRSS) score and reductions in Physician Global Assessment (PGA) from baseline. 66.6% (4/6) subjects experienced reductions in HAQ-DI score relative to baseline. These findings suggested that UCART elicited favorable overall responses in subjects with scleroderma and substantially improved their clinical symptoms and parameters (Figure 9); there was a noticeable alleviation in the involvement of skin, lungs, and other organs (Figures 10A-10C). At the start of cell therapy, all subjects had discontinued immunosuppressants, and the glucocorticoid dose was tapered from a high daily dose (average: 11.1 mg/day, range: 4-20 mg/day) to a low daily maintenance level (average: 4.9 mg/day, range: 0-15 mg/day).

For subjects Sd-01 and Sd-02, skin elasticity was further measured by wave elastography 18, and the elastographic color map from skin elastography revealed a transition from predominantly hard (red) tissue to softer (blue) tissue, indicating reduced stiffness. At M1 the elasticity of skin on the thighs was normal in both patients (Figure 10A). The severity of interstitial lung disease (ILD) was assessed by chest computed tomography (CT) scans, lung function test and serum concentration of KL-6. Improvement of ILD in Sd-01 was revealed by

an increase in FVC from 43.7% to 55% at M3 (Figure 9). Axial and coronal chest high-resolution CT (HRCT) scans for Sd-01 revealed multiple patchy and linear opacities in both lungs, accompanied by thickened interlobular septa (Figure 10B), indicating disseminated inflammatory exudation. At M3 and M6, a significant reduction was observed in bilateral pulmonary inflammation, interlobar effusion, and fibrous strands. The KL-6 decreased from 605 U/mL at baseline to 203 U/mL at M3 (Figure 9). For Sd-02, HRCT scan showed fibrous linear opacities in the posterior basal segment of the right lower lobe and ground-glass opacities, suggesting dispersed inflammatory exudation within the alveoli of the right upper lobe. The inflammation in the right lung was nearly fully resolved at M1, and the low level of inflammation was maintained at M3 and M6.

The cardiac function of Sd-01 and Sd-02 was evaluated by echocardiography. Cardiac fibrotic lesions were measured by cardiac magnetic resonance (CMR). For Sd-01, baseline CMR recovered images with T2-weighted inversion, and mid-wall strip LGE showed local edema with fibrosis formation in the left ventricular wall, which were resolved at M3 (Figure 10B). For Sd-02, baseline CMR showed an elevated native T1 in the left ventricular lateral wall, indicating acute myocardial injury, which was also reduced at M3 and M6 (Figure 10C). After UCART intervention, both patients showed reduced T1 mapping values across all myocardial segments, suggesting diminished cardiac fibrosis (Figure 10C).

Example 14: Evaluation of UCART treatment in Refractory Immune-mediated necrotizing myopathy (IMNM)

Therapeutic Response Evaluation

As is shown in Figure 11A, Figure 11B and Figure 11C, the numbers of B cells decreased and UCART cells were increased remarkably, as well as CAR copies. Meanwhile, the secretion level of cytokine IL-6 was low during the process of treatment.

At baseline and follow-up timepoints, the Total Improvement Score (TIS) was used to evaluate the therapeutic response of the UCAR T cell treatment. It is a composite response criterion based on improvement in six validated core set measures (CSMs), including physician global activity (PGA), Patient Global Activity (PtGA), Manual muscle testing (MMT), Health

Assessment Questionnaire (HAQ) and Extra-muscular Activity (EMDA). The response is defined as a subject with an increase from baseline by improvement category (minimal ≥ 20 , moderate ≥ 40 , and major ≥ 60). The Myositis Disease Activity Assessment Tool (MDAAT) was used to assess activity in extra-muscular organs, such as constitutional, cutaneous, skeletal, gastrointestinal, pulmonary, and cardiac systems. Muscle inflammatory infiltration was evaluated through MRI and thigh muscle biopsy. The levels of anti-SRP autoantibodies were measured using quantitative immunoblotting analysis and then adjusted for serum IgG concentration at each time point (Figure 11A, Figure 11B, Figure 11C, Figure 12A, Figure 12B and Figure 13).

In summary, during evaluation, muscle biopsies showed signs of IMNM with different size of muscle fibers, and scattered necrotic fibers and regenerated muscle fibers could be seen, the kernel fibers were increased, the endomyositis was mild and moderate hyperplasia, and no obvious inflammatory cell infiltration was observed in the intermuscular tissue.

Clinical Results

Subject IMNM-01 was diagnosed with signal recognition particle (SRP)-IMNM, which was characterized by the presence of anti-SRP autoantibodies that target the signal recognition particle, skeletal muscle necrotizing and atrophy on biopsy, and high serum levels of muscle enzymes. IMNM-01 exhibited sustained improvement after treatment with UCART.

Specifically, the total improvement score (TIS) of Subject IMNM-01 was 72.5 at baseline, which increased to 100 after 1 month of treatment and remained at this score during the subsequent follow-up period. Meanwhile, the creatine kinase (CK) level decreased from an initial level of 2295 U/L to 1383 U/L at 1 month, further decreased to 606 U/L at 2 months, and was 234 U/L at 3 months, close to the normal range. Physician global assessment (PGA) and patient global assessment of disease activity (PtGA) also showed significant improvement from baseline after 3 months of treatment. Muscle strength was assessed by manual muscle testing (MMT-8), which showed an improvement from baseline score of 75 (out of 150) to 85 at 1 month, 117 at 2 months, and 145 at 3 months. A significant improvement in quality of life was also noted; the Health Assessment Questionnaire-Disability Index (HAQ-DI) score decreased from 2 at baseline to 1.75 at 1 month and further decreased to 0.25 at 3 months. After 2 months of treatment, extramuscular disease activity showed a significant decrease from

7 to 1 and continued to improve at subsequent follow-ups. Anti-SRP antibodies decreased from 331 U/L at baseline to undetectable levels after 1 month of treatment, and remained undetectable even after B cells were restored to normal range at M6 (Figure 14).

The STIR sequence of magnetic resonance imaging (MRI) indicated a significant reduction in myositis inflammation at 3 months compared to baseline, with evident muscle regeneration and complete resolution of interstitial pneumonia (Figure 15). The subject was bedridden for many years before treatment and regained the ability of independent ambulation and self-care after UCART treatment. All immunosuppressants had been discontinued at the start of cell therapy, and dose of hormonotherapy was also reduced from 100 mg/day before cell infusion to 20 mg/day. It is noteworthy that 8 months after treatment, the subject exhibited elevated levels of creatine kinase and anti-SRP antibodies without experiencing discomfort or requiring an increased dose of glucocorticoid. By the next month, a reduction was seen in these indicators, which suggested sustained therapeutic benefits.

These pathological changes showed significant improvement after CAR T therapy. There were almost no new damaged muscle cells, very little inflammation cell infiltration, and a clear decrease in SRP expression compared to before treatment.

Example 15: Evaluation of UCART treatment in Refractory Systemic Lupus Erythematosus (SLE)

Results

4 subjects were demonstrated significant reductions in their Physician Global Assessment (PGA) scores from baseline. 100% (4/4) of these subjects achieved SRI-4 response, and their Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-2000 or SELEN-SLEDAI) scores significantly decreased from baseline. Among them, 3 subjects had reductions in SLEDAI scores from 18, 14 and 14 at baseline to 0 at M3 or M6, and 1 subject had reductions in SLEDAI scores from 10 at baseline to 2 at M6. All subjects showed improvements in the British Isles Lupus Assessment Group 2004 (BILAG-2004) index to various extents, substantial reductions in ds-DNA and anti-SM antibodies from baseline, and notable increases in complements C3 and C4 from baseline. For 3 subjects whose proteinuria were high and followed-up with 6 months, exhibited significant improvements in urinary protein/creatinine ratio (UPCR) or 24-hour proteinuria, all of them achieved complete response at month 6. These

results indicated that UCART had a positive overall response in subjects with SLE, with significant improvements in clinical symptoms and lab testing (Figures 16 and 17). At the start of cell therapy, all subjects had discontinued immunosuppressants, and the glucocorticoid dose was tapered from a high daily dose at 13.1 mg/day (range, 10-20 mg/day) to a low daily maintenance level at 4.6 mg/day (range: 2.5-10 mg/day).

Example 16: Evaluation of killing activity of UCART on B cells in PBMC from healthy donor

Several samples of PBMC were collected from healthy donors. The total amount of PBMC cells was calculated according to the cell count. The incubation system was prepared. CD19 magnetic sorting beads were added for sorting B cells. Cell killing experiments were performed using the TrPDL1-UCART obtained in Example 6 as effector cells.

The sequence used in the present disclosure is shown in Figure 7.

Example 17: Pharmacokinetic and pharmacodynamic analyses

17.1. Pharmacokinetic Analysis

The expansion and persistence of UCART in subjects were monitored by flow cytometry and qPCR. After UCART infusion, cell expansion occurred in all subjects, and the peak levels in peripheral blood were primarily observed between 7-14 days. The peak concentration of CAR-T cells (mean $\pm$ SD) in all 13 subjects was 79.94 ± 119.43 cells/ μ L. The percentages and counts of CAR-T cells in most subjects increased significantly after UCART infusion, indicating successful *in vivo* expansion of implanted CAR-positive cells and potential for maintenance of prolonged B-cell depletion. At 1, 2, and 3 months of follow-up, CAR-positive cells gradually decreased, with mean percentages of 9.10%, 2.51%, and 0.79%, and mean counts of 42.02 cells/ μ L, 27.80 cells/ μ L, and 18.85 cells/ μ L. For subject IMNM-01 who was followed up to month 9, the CAR-positive cell counts in month 6 and month 9 were 53.75 cells/ μ L and 16.07 cells/ μ L, respectively (Figures 18-19). qPCR demonstrated a consistent change trend between *in vivo* CAR copy numbers and CAR-positive cell counts after UCART infusion, with peak levels occurring between 7-14 days. The peak CAR copy number among the 12 subjects was $15,170 \pm 33,207$ copies/ μ g. The CAR copy number gradually decreased at

1, 2, and 3 months of follow-up (Figure 20). The results demonstrated that UCART can persist *in vivo*.

17.2. Pharmacodynamic Analysis

Consistent with the expansion data of UCART *in vivo*, except for one subject with a short observation period, 12 subjects exhibited a significant decrease in B cells within 7 days after UCART infusion, and basically achieved complete depletion on day 7, which coincided with the *in vivo* expansion time of CAR-T cells. B cell depletion persisted until month 2 or 3, after which B cells gradually recovered. For Subject Sd-01, the B cell count was at a low level (5 cells/ μ L) at baseline, which persisted until month 6. Upon follow-up at month 6, the subject's B cells gradually recovered to 74 cells/ μ L. In summary, following UCART infusion, CAR-T cells expanded, resulting in profound and sustained depletion of B cells in the peripheral blood of the subjects (Figures 21-22).

Example 18: Safety Analysis

The median follow-up time was 148 days (range, 7-293) after UCART administration, and the safety data obtained showed that all subjects who received the UCART treatment were well tolerated. There was no treatment discontinuation or withdrawal due to AE, no CRS grade ≥ 2 , no ICANS and no GvHD. Most AEs in the study were mild to moderate in severity, indicating a manageable safety profile.

18.1. Adverse Events

Based on the summary of AEs in the study, the top 3 SOC for AEs in the 13 subjects were investigations (56.51%), metabolism and nutrition disorders (16.86%), and blood and lymphatic system disorders (9%). In terms of severity, the severity of AEs was mostly (79%) mild to moderate (CTCAE: Grade 1-2). The clinical manifestations of AEs observed in the study were mainly hematological toxicity caused by lymphodepleting drugs and common complications in subjects with autoimmune diseases. After clinical observation or symptomatic treatment, most AEs (72%) were recovered or resolved within an average of 12 days following UCART infusion.

18.2. Adverse Events of Special Interest (AESI)

Regarding the AEs associated with CAR-T cell therapy, the safety of UCART in the treatment of 13 subjects with refractory autoimmune diseases was highly favorable, with only 2 SLE subjects experiencing Grade 1 CRS and no serious infection (Grade 3/4), no ICANS and GvHD observed in the 13 subjects.

18.3. CRS

In this study, only 2 subjects with refractory SLE (SLE-04/SLE-05) developed Grade 1 CRS on D4 and D5, respectively, with clinical symptom of pyrexia (body temperature up to 39.1°C), and the remaining vital signs were stable, with no hypotension or hypoxemia. Overall, UCART was well tolerated in subjects with refractory autoimmune diseases. Among the 13 subjects treated with UCART, only 2 subjects experienced grade 1 CRS; ICANS or GvHD was not observed in all subjects. Although B cells were gradually exhausted starting from day 6 to 8 after UCART infusion, no treatment-related hypogammaglobulinemia or serious infections were observed in the study, indicating a favorable safety profile of UCART in subjects with refractory autoimmune diseases compared with the toxicities reported for CAR-T cell therapy in hematological malignancies. Additionally, a review of AEs observed in the project revealed that the most common AEs were primarily hematological toxicities and laboratory abnormalities, consistent with the known adverse reactions to lymphodepleting drugs and complications of autoimmune diseases. During the study, the AEs possibly related to UCART, such as lymphocyte count decreased and white blood cell count decreased, were mostly reversible after symptomatic treatment.

Conclusion

Regarding efficacy, one subject with idiopathic myopathy showed sustained improvement during the 9-month monitoring period after treatment with UCART. All 4 subjects with SLE achieved SRI-4 response after treatment. All 6 scleroderma subjects' CRIS scores exceeded 0.6 after 3 months of treatment; 5 of them showed improvements in FVC percentage from baseline; all 6 subjects exhibited a significant decrease in modified Rodnan skin score (mRSS) from baseline, indicating significant improvement in all subjects.

In addition, UCART continuously and steadily expanded in treated subjects, reaching a significant peak around day 14, and UCART cells were still detectable in the peripheral blood 3 months after treatment. Consistent with the PK data of UCART *in vivo*, except for 1 subject

with a short observation period, 12 subjects exhibited a significant decrease in B cells within 7 days after UCART infusion, and basically achieved complete depletion on day 7. This depletion persisted for approximately two months, indicating that with the *in vivo* expansion of UCART, a sustained and profound depletion of B cells was achieved in subjects with refractory autoimmune diseases.

The results of this IIT study showed that UCART achieved rapid, significant, and long-lasting expansion in subjects, accompanied by a profound and sustained depletion of B cells. During the treatment, the subjects experienced minor adverse reactions and well tolerated UCART. There was a noticeable improvement in clinical assessment, with serology remission and organs function recovery for all subjects with refractory scleroderma, systemic lupus erythematosus and myositis.

Claims

1. A method for preventing and/or treating a condition and/or a disease in a subject in need thereof, comprising administering to the subject an effective amount of a modified cell and/or a pharmaceutical composition comprising the same;
wherein the condition or the disease is the condition or the disease of a diffuse connective tissue disease or lymphatic malignancies;
wherein the modified cell is, relative to an unmodified counterpart cell:
deficient in an endogenous major histocompatibility complex and an endogenous inhibitory checkpoint molecule;
engineered to express a ligand of the inhibitory checkpoint molecule;
wherein the ligand is a variant of the ligand having increased capability of inducing immunoinhibitory signaling relative to the cognate ligand.
2. The method of claim 1, wherein the modified cell is derived from any one of a somatic cell, a stem cell, and the combination thereof; the somatic cell is preferably an immune cell;
and/or, the modified cell is an animal cell or a human cell;
preferably, the modified cell is allogeneic with reference to the cell of the subject.
3. The method of claim 1 or 2, wherein the unmodified cell is an immune cell, and the immune cell is any one of a T cell, Nature Killer cell, NKT cell, B cell, macrophage cell, monocyte, dendritic cell, neutrophil, and the combination thereof; or,
the unmodified cell is a stem cell, and the stem cell is a hematopoietic progenitor cell, hematopoietic stem cell, CD34⁺ cell, embryonic stem cell, a mesenchymal stem cell or iPSC cell;
preferably, the T cell is any one of $\alpha\beta$ T cell, $\gamma\delta$ T cell, helper T cell, regulatory cell, and the combination thereof; and/or, the T cell is activated T cell.
4. The method of any one of claims 1-3, wherein the endogenous major histocompatibility

complex is a human major histocompatibility complex or a murine major histocompatibility complex;

preferably, the human major histocompatibility complex comprises human leukocyte antigen class I; and/or, the murine major histocompatibility complex comprises histocompatibility-2 class I;

more preferably, the gene of human leukocyte antigen class I is HLA-A, HLA-B, HLA-C, HLA-E or the combination thereof; and/or, the gene of histocompatibility-2 class I is H-2K, H-2D, or the combination thereof.

5. The method of any one of claims 1-4, wherein the endogenous inhibitory checkpoint molecule is any one or more of PD-1, TIM3, TIGIT, LAG3, A2AR, BTLA (CD272), CTLA-4 (CD152), IDO1, IDO2, TDO, KIR, NOX2, VISTA, SIGLEC7 (CD328), PVR(CD155), and SIGLEC9 (CD329);

and/or, the ligand is any one or more of PD-L1, PD-L2, HMGB1, Ceacam-1, phosphatidyl serine (PS), LSECtin, α -synuclein, FGL1, adenosine, HVEM (Herpesvirus Entry Mediator), CD28, B7-H3 (CD276), B7-H4 (VTCN1), PVR(CD155), HLA class I, sialoglycoprotein, CD112, CD113, Galectin9, CD24, and CD47.

6. The method of any one of claims 1-5, wherein the variant comprises at least one mutation that increases the capability of inducing immunoinhibitory signaling;

preferably, the mutation is any one of substitution, deletion, insertion, and any combination thereof;

more preferably, the mutation is a deletion of at least one amino acid residue.

7. The method of any one of claims 1-6, wherein the variant lacks at least part of an extracellular domain, a transmembrane domain and/or an intracellular domain relative to the cognate inhibitory checkpoint molecule or the ligand thereof;

preferably, the inhibitory checkpoint molecule is PD-1; and the ligand thereof is PD-L1;

more preferably, the variant of the ligand has the amino acid sequence of SEQ ID NO: 7 or the

amino acid sequence having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8% or at least 99.9% identity with SEQ ID NO: 7.

8. The method of any one of claims 1-7, wherein the modified cell is further engineered to comprise a chimeric antigen receptor or a T cell receptor;

preferably, the CAR comprises an antigen binding domain, a transmembrane domain, and an intracellular signaling domain, further optionally the CAR further comprises a costimulatory signaling region;

more preferably, the antigen is selected from BCMA, CD7, CD10, CD19, CD20, CD22, CD24, CD30, CD33, CD34, CD38, CD44, CD79a, CD79b, CD123, CD138, CD 179b, CEA, CLEC12A, Claudin18.2, CS-1, DLL3, EGFR, EGFRvIII, EPCAM, FLT-3, FOLR1, FOLR3, GD2, gpA33, GPC3, HER2, HM1.24, LGR5, Mesothelin, MSLN, MCSP, MICA/B, PSMA, PAMA, P-cadherin, and ROR1; e.g., the antigen is CD19.

9. The method of any one of claims 1-8, wherein the modified cell or the pharmaceutical thereof is administered independently or in combination;

and/or, the diffuse connective tissue disease comprises systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis (RA), idiopathic inflammatory myopathy (IIM), Churg-Strauss Syndrome and microscopic polyangitis; preferably, the idiopathic inflammatory myopathy comprises polymyositis (PM), dermatomyositis (DM), amyopathic dermatomyositis (ADM), immune necrotizing myopathy (IMNM), inclusion body myositis (IBM) and juvenile dermatomyositis (JDM);

and/or, the lymphatic malignancies comprises B cell lymphoma and B lymphocyte leukemia; preferably, the B cell lymphoma comprises Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL), B lymphocyte leukemia comprises acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

10. The method of any one of claims 1-9, wherein the modified cell is prepared and/or expanded

in vitro, *in vivo* or *ex vivo*;

and/or, the subject in need thereof is a subject received at least 1, 2, 3 or 4 immunosuppressive therapy; preferably, the therapy is a therapy comprising one or more selected from glucocorticoids, hydroxychloroquines, mycophenolate mofetil tacrolimus, cyclophosphamide, azathioprine, tocilizumab, methotrexate, telitacicept, belimumab and rituximab.

11. A modified cell or a population thereof, wherein the modified cell is the modified cell defined in any one of claims 1-10.

12. A polynucleotide targeting a major histocompatibility complex, wherein the polynucleotide is capable of inactivating or weakening the expression of a major histocompatibility complex; preferably, the major histocompatibility complex is major histocompatibility complex class I; and/or, the polynucleotide comprises an sgRNA;

more preferably, the major histocompatibility complex is human major histocompatibility complex, *e.g.* HLA-A, HLA-B, HLA-C and/or HLA-E; and/or, the sgRNA targets any one or more of the DNA sequence of SEQ ID NOs: 29-45 and 73-168.

13. A method for producing the modified cell of claim 11, comprising the step of introducing the polynucleotide of claim 10 and a gene editing system into an unmodified counterpart cell; preferably, the gene editing system is a CRISPR system.

14. A pharmaceutical composition comprising (1) the modified cell of claim 11, and/or the polynucleotide of claim 12, and (2) a pharmaceutically acceptable carrier and/or excipients.

15. A kit comprising the modified cell of claim 11 or the pharmaceutical composition of claim 14.

16. The modified cell or a population thereof of claim 11, the polynucleotide of claim 12, the pharmaceutical composition of claim 14, and the kit of claim 15 for use in preventing and/or treating a condition and/or a disease of a diffuse connective tissue disease; preferably, the diffuse connective tissue disease comprises systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis (RA), idiopathic inflammatory myopathy (IIM), Churg-Strauss

Syndrome and microscopic polyangitis; more preferably, the idiopathic inflammatory myopathy comprises polymyositis (PM), dermatomyositis (DM), amyopathic dermatomyositis (ADM), immune necrotizing myopathy (IMNM), inclusion body myositis (IBM) and juvenile dermatomyositis (JDM);

and/or,

preferably, the lymphatic malignancies comprises B cell lymphoma and B lymphocyte leukemia; more preferably, the B cell lymphoma comprises Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL), B lymphocyte leukemia comprises acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

17. A use of the modified cell or a population thereof of claim 11, the polynucleotide of claim 12, the pharmaceutical composition of claim 14, and the kit of claim 15 in preparing a medicament for preventing and/or treating a condition and/or a disease of a diffuse connective tissue disease;

preferably, the diffuse connective tissue disease comprises systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis (RA), idiopathic inflammatory myopathy (IIM), Churg-Strauss Syndrome and microscopic polyangitis; more preferably, the idiopathic inflammatory myopathy comprises polymyositis (PM), dermatomyositis (DM), amyopathic dermatomyositis (ADM), immune necrotizing myopathy (IMNM), inclusion body myositis (IBM) and juvenile dermatomyositis (JDM);

and/or,

preferably, the lymphatic malignancies comprises B cell lymphoma and B lymphocyte leukemia; more preferably, the B cell lymphoma comprises Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL), lymphocyte leukemia comprises acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL).