

NON-HLA RESTRICTED T CELL RECEPTORS AND USES THEREOF**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims priority to U.S. Provisional Patent Application Serial No. 62/629,072, filed February 11, 2018, the content of which is incorporated by reference in its entirety, and to which priority is claimed.

INTRODUCTION

The presently disclosed subject matter provides methods and compositions for enhancing the immune response toward cancers and pathogens. It relates to novel designs of T cell receptors (TCRs) and engineered immunoresponsive cells comprising the same. The engineered immunoresponsive cells comprising the novel TCRs are antigen-directed.

BACKGROUND OF THE INVENTION

Adoptive immunotherapy using antigen recognizing receptors (e.g., chimeric antigen receptors (CARs)) has shown remarkable clinical results in the treatment of leukemia and is one of the most promising new strategies to treat cancer. To generate CAR therapies, current clinical protocols employ autologous T cells and randomly integrating vectors, including gamma-retroviral, lentiviral and transposons, which all result in semi-random integration and variable expression of the CAR owing to transgene variegation. Altogether, the conjunction of autologous cell sourcing and random vector integration is prone to generating cell products with variable potency. Thus, there is a need of novel designs of antigen recognizing receptors having consistent potency and increased ability to detect low levels of target antigen.

SUMMARY OF THE INVENTION

The presently disclosed subject matter generally provides HLA-independent (or non-HLA restricted) T cell receptors (referred to as "HI-TCRs") that bind to an antigen of interest in an HLA-independent manner and immunoresponsive cells comprising thereof. The presently disclosed subject matter also provides methods of using such cells for inducing and/or enhancing an immune response to a target antigen, and/or treating and/or preventing neoplasia or other diseases/disorders where an increase in an antigen-specific immune response is desired.

The presently disclosed subject matter provides recombinant T cell receptor (TCR) comprising an antigen binding chain that comprises an extracellular antigen-binding domain and a constant domain, wherein the recombinant TCR binds to an antigen in an HLA-independent manner.

5 In certain embodiments, the constant domain comprises a native or modified TRAC peptide, and/or a native or modified TRBC peptide. In certain embodiments, the constant domain is capable of forming a homodimer or a heterodimer with another constant domain.

10 In certain embodiments, the recombinant TCR is expressed from an expression cassette placed in an endogenous *TRAC* locus and/or a *TRBC* locus of an immunoresponsive cell. In certain embodiments, the placement of the recombinant TCR expression cassette disrupts or abolishes the endogenous expression of a TCR comprising a native TCR α chain and/or a native TCR β chain in the immunoresponsive cell. In certain embodiments, the placement of the recombinant TCR expression cassette prevents or eliminates mispairing between the recombinant TCR and a native TCR α chain and/or a native TCR β chain in the immunoresponsive cell. In certain embodiments, the antigen binding chain is capable of associating with a CD3 ζ polypeptide. The antigen binding chain, upon binding to an antigen, is capable of activating the CD3 ζ polypeptide associated to the antigen binding chain. The activation of the CD3 ζ polypeptide is capable of activating an immunoresponsive cell. The CD3 ζ polypeptide can be endogenous or exogenous. In certain embodiments, the CD3 ζ polypeptide is endogenous and is endogenous and integrated in the native CD3 complex. In certain embodiments, the CD3 ζ polypeptide is exogenous and optionally integrated with a co-stimulatory molecule selected from the group consisting of a CD28 polypeptide, a 4-1BB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a DAP-10 polypeptide and any combination thereof. In certain embodiments, the antigen binding chain further comprises a co-stimulatory region, wherein the recombinant TCR, upon binding to an antigen, is capable of stimulating an immunoresponsive cell. The co-stimulatory region can include a co-stimulatory molecule selected from the group consisting of a CD28 polypeptide, a 4-1BB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a DAP-10 polypeptide and any combination thereof. In certain embodiments, the co-stimulatory region comprises a CD28 polypeptide.

 In certain embodiments, the recombinant TCR is capable of associating with a CD3 complex. In certain embodiments, the recombinant TCR is capable of integrating

with a CD3 complex and providing HLA-independent antigen recognition. In certain embodiments, the CD3 complex is endogenous. In certain embodiments, the recombinant TCR replaces an endogenous TCR in a CD3/TCR complex.

In certain embodiments, the extracellular antigen-binding domain is capable of dimerizing with another extracellular antigen-binding domain. The extracellular antigen-binding domain can include a ligand for a cell-surface receptor, a receptor for a cell surface ligand, an antigen binding portion of an antibody or a fragment thereof or an antigen binding portion of a TCR. In certain embodiments, the extracellular antigen-binding domain comprises a heavy chain variable region (V_H) of an antibody, or a VHH from a camelid V_H -only antibody and/or a light chain variable region (V_L) of an antibody. In certain embodiments, the extracellular antigen-binding domain is capable of dimerizing with another extracellular antigen-binding domain. In certain embodiments, the extracellular antigen-binding domain comprises a V_H of an antibody, wherein the human, murine, or camelid V_H is capable of dimerizing with another extracellular antigen-binding domain comprising a V_L of the antibody and form a fragment variable (Fv). In certain embodiments, the extracellular antigen-binding domain comprises a V_L of an antibody, wherein the V_L is capable of dimerizing with another extracellular antigen-binding domain comprising a V_H of the antibody and form a fragment variable (Fv).

In certain embodiments, the recombinant TCR binds to a tumor antigen. The tumor antigen can be selected from the group consisting of CD19, MUC16, MUC1, CAIX, CEA, CD8, CD7, CD10, CD20, CD22, CD30, CLL1, CD33, CD34, CD38, CD41, CD44, CD49f, CD56, CD74, CD133, CD138, EGP-2, EGP-40, EpCAM, erb-B2,3,4, FBP, Fetal acetylcholine receptor, folate receptor-a, GD2, GD3, HER-2, hTERT, IL-13R-a2, K-light chain, KDR, LeY, L1 cell adhesion molecule, MAGE-A1, Mesothelin, ERBB2, MAGEA3, p53, MART1, GP100, Proteinase3 (PR1), Tyrosinase, Survivin, hTERT, EphA2, NKG2D ligands, NY-ES0-1, oncofetal antigen (h5T4), PSCA, PSMA, ROR1, TAG-72, VEGF-R2, WT-1, BCMA, CD123, CD44V6, NKCS1, EGF1R, EGFR-VIII, and CD99, CD70, ADGRE2, CCR1, LILRB2, LILRB4, PRAME, and ERBB. In certain embodiments, the tumor antigen is CD19.

In certain embodiments, the recombinant TCR exhibits greater antigen sensitivity than a CAR targeting the same antigen. In certain embodiments, the recombinant TCR is capable of inducing an immune response when binds to an antigen that has a low density

on the surface of a tumor cell. In certain embodiments, the antigen that has a low density on the cell surface has below about 2,000 molecules per cell.

The presently disclosed subject matter further provides immunoresponsive cells comprising a recombinant TCR described herein. In certain embodiments, the expression cassette of at least one antigen binding chain of the recombinant TCR is placed at an endogenous gene locus of the immunoresponsive cell. In certain embodiments, the expression cassettes of two antigen binding chains of the recombinant TCR are placed at an endogenous gene locus of the immunoresponsive cell, wherein the two antigen binding chains are capable of dimerization. The placement of the expression cassette of the recombinant TCR can disrupt or abolish the endogenous expression of a TCR comprising a native TCR α chain and/or a native TCR β chain in the immunoresponsive cell, whereby preventing or eliminating mispairing between the recombinant TCR and a native TCR α chain and/or a native TCR β chain in the immunoresponsive cell. The endogenous gene locus can be a *CD3 δ* locus, a *CD3 ϵ* locus, a *CD247* locus, a *B2M* locus, a *TRAC* locus, a *TRBC* locus or a *TRGC* locus or a *TRDC* locus. In certain embodiments, the endogenous gene locus is a *TRAC* locus and/or a *TRBC* locus. The endogenous gene locus can include a modified transcription terminator region. In certain embodiments, the modified transcription terminator region comprises a genomic element selected from the group consisting of a TK transcription terminator, a GCSF transcription terminator, a TCRA transcription terminator, an HBB transcription terminator, a bovine growth hormone transcription terminator, a SV40 transcription terminator and a P2A element; the P2A element allows the use of the endogenous transcription terminator of the targeted gene. In certain embodiments, when one endogenous T cell receptor locus in a cell is modified to express the at least one antigen binding chain of the recombinant TCR, one or more other endogenous T cell receptor locus in the cell is modified to eliminate the expression of an endogenous TCR chain. In certain embodiments, the one or more other endogenous T cell receptor loci are further modified to express a gene of interest. The gene of interest can be an anti-tumor cytokine, a co-stimulatory molecule ligand, a tracking gene or a suicide gene. In certain embodiments, one or more endogenous TCR loci are further modified to incorporate a sequence encoding co-stimulatory signaling domain(s) to generate a TCR chain containing such signaling domain(s) at the carboxy terminus.

In certain embodiments, the immunoresponsive cell is selected from the group consisting of a T cell, a cytotoxic T lymphocyte (CTL), a regulatory T cell, a Natural Killer T (NKT) cell, a human embryonic stem cell, and a pluripotent stem cell from

which lymphoid cells may be differentiated. In certain embodiments, the immunoresponsive cell is autologous.

In certain embodiments, the immunoresponsive cell further comprises at least one exogenous co-stimulatory ligand. In certain embodiments, the co-stimulatory ligand is selected from the group consisting of CD80, CD86, 41BBL, CD275, CD40L, OX40L and any combination thereof. In certain embodiments, the cell further comprises or consists of one exogenous co-stimulatory ligand. In certain embodiments, the one exogenous co-stimulatory ligand is CD80 or 4-1BBL. In certain embodiments, the cell further comprises or consists of two exogenous co-stimulatory ligands. In certain embodiments, the two exogenous co-stimulatory ligands are CD80 and 4-1BBL.

In certain embodiments, the immunoresponsive cell further comprises at least one chimeric costimulatory receptor (CCR). In certain embodiments, the CCR comprising a co-stimulatory molecule selected from the group consisting of a CD28 polypeptide, a 4-1BB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a DAP-10 polypeptide and any combination thereof.

The presently disclosed subject matter also provides pharmaceutical compositions comprising the immunoresponsive cell(s) disclosed herein and a pharmaceutically acceptable excipient. The pharmaceutical composition can be used for treating a neoplasia.

Further provided are methods of reducing tumor burden in a subject. In addition, the presently disclosed subject matter provides methods of lengthening survival of a subject having a neoplasm (e.g., cancer). In certain embodiments, the method comprises administering to the subject an effective amount of the immunoresponsive cells described herein or the pharmaceutical composition described herein.

The presently disclosed subject matter also provides methods of treating or preventing a neoplasm. In certain embodiments, the method comprises administering to the subject an effective amount of the immunoresponsive cells described herein or the pharmaceutical composition described herein. The neoplasm can be selected from the group consisting of blood cancer, B cell leukemia, multiple myeloma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, non-Hodgkin's lymphoma and adenocarcinoma. In certain embodiments, the neoplasm is B cell leukemia, multiple myeloma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, or non-Hodgkin's lymphoma, and the recombinant TCR binds to CD19. In

certain embodiments, the neoplasm is B cell leukemia, multiple myeloma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, an adenocarcinoma, or non-Hodgkin's lymphoma, and the recombinant TCR binds to CD19, MUC16, MUC1, CAIX, CEA, CD8, CD7, CD10, CD20, CD22, CD30, CLL1, CD33, CD34, CD38, CD41, CD44, CD49f, CD56, CD74, CD133, CD138, EGP-2, EGP-40, EpCAM, erb-B2,3,4, FBP, Fetal acetylcholine receptor, folate receptor-a, GD2, GD3, HER-2, hTERT, IL-13R-a2, K-light chain, KDR, LeY, L1 cell adhesion molecule, MAGE-A1, Mesothelin (MSLN), ERBB2, MAGEA3, p53, MART1, GP100, Proteinase3 (PR1), Tyrosinase, Survivin, hTERT, EphA2, NKG2D ligands, NY-ES0-1, oncofetal antigen (h5T4), PSCA, PSMA, ROR1, TAG-72, VEGF-R2, WT-1, BCMA, CD123, CD44V6, NKCS1, EGF1R, EGFR-VIII, CD99, CD70, ADGRE2, CCR1, LILRB2, LILRB4, PRAME, and ERBB. In certain embodiments, the neoplasm is CD19+ ALL.

The presently disclosed subject matter further provides methods for producing an antigen-specific immunoresponsive cell. In certain embodiments, the method comprises introducing into an immunoresponsive cell a nucleic acid sequence encoding a recombinant TCR described herein. The nucleic acid sequence can be comprised in a vector. In certain embodiments, the expression cassette of at least one antigen binding chain of the recombinant TCR is placed at an endogenous gene locus of the immunoresponsive cell. In certain embodiments, the expression cassettes of two antigen binding chains of the recombinant TCR are placed at an endogenous gene locus of the immunoresponsive cell, wherein the two antigen binding chains are capable of dimerization. The endogenous gene locus can be a *CD3 δ* locus, a *CD3 ϵ* locus, a *CD247* locus, a *B2M* locus, a *TRAC* locus, a *TRBC* locus, a *TRDC* locus and/or a *TRGC* locus. In certain embodiments, the endogenous gene locus is a *TRAC* locus or a *TRBC* locus. In certain embodiments, the placement of the expression cassette of the recombinant TCR disrupts or abolishes the endogenous expression of a TCR comprising a native TCR α chain and/or a native TCR β chain in the immunoresponsive cell, whereby preventing or eliminating mispairing between the recombinant TCR and a native TCR α chain and/or a native TCR β chain in the immunoresponsive cell. In certain embodiments, the endogenous gene locus comprises a modified transcription terminator region. In certain embodiments, the modified transcription terminator region comprises a genomic element selected from the group consisting of a TK transcription terminator, a GCSF transcription terminator, a TCRA transcription terminator, an HBB transcription terminator, a bovine growth hormone transcription terminator, an SV40 transcription

terminator and a P2A element. In certain embodiments, when one endogenous T cell receptor locus in a cell is modified to express the at least one antigen binding chain of the recombinant TCR, one or more other endogenous T cell receptor locus in the cell is modified to eliminate the expression of an endogenous TCR chain. In certain
5 embodiments, the one or more other endogenous T cell receptor locus are further modified to express a gene of interest. In certain embodiments, the gene of interest is an anti-tumor cytokine, a co-stimulatory molecule ligand, a tracking gene or a suicide gene.

 The presently disclosed subject matter further provides nucleotide acids encoding a recombinant TCR described herein, and nucleic acid compositions comprising a
10 recombinant TCR described herein. In certain embodiments, the nucleic acid sequences are comprised in a vector. The presently disclosed subject matter also provides vectors comprising the nucleic acid composition described herein. Further provided are kits comprising a recombinant TCR described herein, an immunoresponsive cell described herein, a pharmaceutical composition described herein, a nucleic acid composition
15 described herein, or a vector described herein. In certain embodiments, the kit further comprises written instructions for treating and/or preventing a neoplasm, a pathogen infection, an autoimmune disorder, or an allogeneic transplant.

 Illustrative neoplasms for which the presently disclosed subject matter can be used include, but are not limited to leukemias (e.g., acute leukemia, acute lymphocytic
20 leukemia, acute myelocytic leukemia a, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, acute monocytic leukemia, acute erythroleukemia, chronic leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia), polycythemia vera, lymphoma (Hodgkin's disease, non-Hodgkin's disease), Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors such as
25 sarcomas and carcinomas (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma,
30 adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, nile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, uterine cancer, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder

carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, schwannoma, meningioma, melanoma, neuroblastoma, and retinoblastoma).

- 5 The presently disclosed subject matter further provides use of any recombinant TCR, any pharmaceutical composition or any immunoresponsive cell disclosed herein for used in a therapy.

BRIEF DESCRIPTION OF THE FIGURES

- 10 The following Detailed Description, given by way of example, but not intended to limit the presently disclosed subject matter to specific embodiments described, may be understood in conjunction with the accompanying drawings.

- Figures 1A-1E depict HLA-Independent TCR-based Chimeric Antigen Receptor HIT (HIT-CAR, i.e., HI-TCR or HIT) and gene targeting strategy at the TRAC locus in human T cells. (A) Schematic representation of the T Cell Receptor (TCR), the B Cell Receptor (BCR), a Chimeric Antigen Receptor (CAR) and the HLA-Independent TCR-based Chimeric Antigen Receptor (HIT-CAR, i.e., HI-TCR or HIT). (B) CRISPR/Cas9-targeted integration of the 3 receptors into the TRAC locus. Top: TRAC locus; middle: rAAV6 containing the different receptor cassette flanked by homology arms. (C) Representative TCR/Mouse F(ab')₂ flow cytometry plots 4 days after TRAC targeting. The TCR antibody epitope recognize the constant chain of the TCR alpha and beta. (D) Cytotoxic activity using an 18-hour bioluminescence assay, using firefly luciferase (FFL)-expressing NALM-6 as targets cells (n=3). (E) Relative CAR MFI (1=MFI at 0h) of CAR T cells after 1, 2 or 4 (arrows) stimulations on CD19 positive target cells.

- Figures 2A-2B depict expression and therapeutic efficacy of HI-TCR. (A) Representative TCR/Mouse F(ab')₂ flow cytometry plots 4 days after TRAC targeting. (B) Kaplan-Meier analysis of the mice survival where NALM-6- bearing mice were treated with 5x10⁵ CAR T cells.

- Figures 3A-3E depict gene targeting strategy and expression of NYESO TCR. (A) Schematic representation of the NYESO TCR genes integrated into the TCR alpha or beta chain. (B) Representative TCR-V-beta-1 flow cytometry plots 4 days after TRAC or TRBC targeting. (C) Cytotoxic activity using an 18-hour bioluminescence assay, using firefly luciferase (FFL)-expressing PC3 as targets cells (n=3). (D) Schematic representation of a co-targeting into both the TCR alpha and the TCR beta. (E)

Representative TCR-V-beta-1/4-1BBL flow cytometry plots 4 days after TRAC and TRBC co- targeting.

Figures 4A-4C depict strategy of integrating a CAR into a TRAC locus and modulation the expression by various transcriptional termination signals / 3' untranslated regions (3'UTR). (A) Schematic representation of the 1928z CAR gene integrated into the TRAC locus. Poly A (black box) corresponds to the segment of the CAR cassette that was modified to test different viral and mammalian 3' UTRs. (B) Representative CAR flow plots 3 days after TRAC (left panel).and geometric mean fluorescence intensity (gMFI), MFI, and Median values for the CAR-expressing population (right panel). Boxed is the original 3'UTR sequence of bovine growth hormone poly A. (C) Absolute (top) and relative (bottom) CAR MFI (1=MFI at 0h) of CAR T cells after 1, 2 stimulations on CD19 positive target cells (as shown in Fig. 1).

Figures 5A and 5B depict efficacy of genetically integrated CARs with different 3'UTR sequences. (A) FFL-NALM-6-bearing mice were treated with 1×10^5 CAR T cells, where tumor burden is shown as bioluminescent signal quantified per animal 14 days post T-cell injection. n = 6 mice per group. (B) Tumor burden (average radiance) of NALM-6-bearing mice treated with 1×10^5 CAR T cells (n = 6; line = one mouse) quantified at days 7, 14, and 21 post T-cell injection.

Figures 6A-6C depict HIT gene targeting at the TRAC locus in human T cells. (A) CRISPR/Cas9-targeted CAR or HIT gene integration into the TRAC locus. Top, TRAC locus; middle, rAAV6 containing the CAR cassette flanked by homology arms; bottom, rAAV6 containing the HIT cassette flanked by homology arms. (B) Representative CAR/HIT flow plots 4 days after transfection of T cells with Cas9 mRNA and TRAC gRNA and addition of AAV6. CAR and HIT surface protein were detected using a goat anti-mouse IgG. (C) Average CAR/HIT mean fluorescence intensity (MFI) analyzed by FACS 4 days after transduction (n = 6 independent experiments).

Figures 7A-7B depict HIT T cells outperform CAR T cells at killing target cells expressing low antigen levels. Nalm6 cell line (expressing firefly luciferase) was gene edited at the CD19 locus using CRISPR/Cas9, and clones expressing different CD19 levels were generated. (A) FACS analysis of representative Nalm6 clones for each CD19 expression level group (Neg = negative). (B) Cytotoxic activity using an 4h bioluminescence assay, using NALM6 as targets cells expressing different CD19 levels and CAR (red squares) or HIT (blue circles) T cells at 1:1 effector (E): target (T) ratio.

Figures 8A-8C depict HIT T cells outperform CAR T cells at killing target cells expressing low antigen levels. Cytotoxic activity using an 18 h bioluminescence assay, using NALM6 as targets cells expressing different CD19 levels (indicated at the right), which were incubated with untransduced T cells (A), CAR T cells (B), and HIT T cells (C) at different effector (E): target (T) ratios.

Figures 9A-9D depict HIT T cells expressing costimulatory ligands outperform CAR T cells in controlling established B-ALL tumor with very low CD19 levels. NALM-6-bearing mice were treated with 4×10^5 untransduced (NT), CAR, or HIT T cells. Tumor burden^[SEP] was quantified weekly over a 54-day period using BLI. Quantification is the average photon count of ventral and dorsal acquisitions per animal at all given time points. Each line represents one mouse, and $n = 5$ mice per group. (A) Untransduced (black) vs CAR (red) T cells. (B) CAR (red) vs HIT (green) T cells. (C) T cells expressing HIT alone (green), HIT + CD80 co-stimulatory ligand (orange), HIT + 41BBL co-stimulatory ligand (pink), or HIT + CD80 + 41BBL co-stimulatory ligands (blue). (D) Mouse survival analysis.

Figures 10A-10C depict that baseline TRAC-CAR expression can be controlled by distinct 3'UTR sequences without affecting cell surface replenishment kinetic after antigen encounter. (A) CRISPR/Cas9-targeted CAR gene integration into the TRAC locus. The targeting construct (AAV) contains the 1928z CAR coding sequence followed by a 3'UTR sequence, flanked by sequences homologous to the TRAC locus (LHA^[SEP] and RHA, left and right homology arm). (B) Each 3'UTR sequence provides different CAR surface levels (measured by FACS). TK thymidine kinase (short version); GCSF: human GCSF, derived from pEF-BOS plasmid; TCRA: TCR alpha, exon 4; HBB: human B-globin; RBG: rabbit B-globin; SV40: simian virus 40 poly A; P2A: porcine teschovirus-1 self-cleaving 2A sequence; it allowed the use of the endogenous TRAC poly A sequence. (C) CAR T cells were stimulated once (indicated by a red arrow) with CD19-expressing 3T3 cells, and CAR MFI was measured every 24h over a 3-day period. All CAR T cells show similar CAR expression regulation.

DETAILED DESCRIPTION OF THE INVENTION

The presently disclosed subject matter provides HLA-independent (or non-HLA restricted) T cell receptors (referred to as "HI-TCRs") that bind to an antigen of interest in an HLA-independent manner. In certain embodiments, the HI-TCRs are TCR molecules where the TCR variable domain is replaced by the variable domain from an antibody

(Fv), resulting in a FvTCR. In certain non-limiting embodiments, the HI-TCR can bind to a tumor antigen or a pathogen antigen. The presently disclosed subject matter also provides cells, including genetically modified immunoresponsive cells (e.g., T cells, NKT cells, or CTL cells) comprising the presently disclosed HI-TCR. In certain non-limiting
5 embodiments, binding of the antigen by the HI-TCR is capable of activating the immunoresponsive cell. The presently disclosed subject matter also provides methods of using such cells for inducing and/or enhancing an immune response to a target antigen, and/or treating and/or preventing neoplasia or other diseases/disorders where an increase in an antigen-specific immune response is desired.

10 **1. Definitions**

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

20 As used herein, the term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, “about” can mean within 3 or more than 3 standard deviations, per the practice in the art. Alternatively, “about” can mean a range of up to
25 about 20%, e.g., up to about 10%, up to about 5%, or up to about 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, e.g., within about 5-fold or within about 2-fold, of a value.

By “activates an immunoresponsive cell” is meant induction of signal transduction
30 or changes in protein expression in the cell resulting in initiation of an immune response. For example, when CD3 Chains cluster in response to ligand binding and immunoreceptor tyrosine-based inhibition motifs (ITAMs) a signal transduction cascade is produced. In certain embodiments, when an endogenous TCR or an exogenous CAR binds to an antigen, a formation of an immunological synapse occurs that includes

clustering of many molecules near the bound receptor (e.g. CD4 or CD8, CD3 $\gamma/\delta/\epsilon/\zeta$, etc.). This clustering of membrane bound signaling molecules allows for ITAM motifs contained within the CD3 chains to become phosphorylated. This phosphorylation in turn initiates a T cell activation pathway ultimately activating transcription factors, such as NF- κ B and AP-1. These transcription factors induce global gene expression of the T cell to increase IL-2 production for proliferation and expression of master regulator T cell proteins in order to initiate a T cell mediated immune response.

By “stimulates an immunoresponsive cell” is meant a signal that results in a robust and sustained immune response. In various embodiments, this occurs after immune cell (e.g., T-cell) activation or concomitantly mediated through receptors including, but not limited to, CD28, CD137 (4-1BB), OX40, CD40 and ICOS. Receiving multiple stimulatory signals can be important to mount a robust and long-term T cell mediated immune response. T cells can quickly become inhibited and unresponsive to antigen. While the effects of these co-stimulatory signals may vary, they generally result in increased gene expression in order to generate long lived, proliferative, and anti-apoptotic T cells that robustly respond to antigen for complete and sustained eradication.

The term “antigen recognizing receptor” as used herein refers to a receptor that is capable of activating an immune or immunoresponsive cell (e.g., a T-cell) in response to its binding to an antigen. Non-limiting examples of antigen recognizing receptors include native or endogenous T cell receptors (“TCRs”), and chimeric antigen receptors (“CARs”).

As used herein, the term “antibody” means not only intact antibody molecules, but also fragments of antibody molecules that retain immunogen-binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. Accordingly, as used herein, the term “antibody” means not only intact immunoglobulin molecules but also the well-known active fragments F(ab')₂, and Fab. F(ab')₂, and Fab fragments that lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)). As used herein, antibodies include whole native antibodies, bispecific antibodies; chimeric antibodies; Fab, Fab', single chain V region fragments (scFv), fusion polypeptides, and unconventional antibodies. In certain embodiments, an antibody is a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised

of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant (C_H) region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant C_L region. The light chain constant region is comprised of one domain, C_L . The V_H and V_L regions can be further sub-divided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1 q) of the classical complement system.

As used herein, “CDRs” are defined as the complementarity determining region amino acid sequences of an antibody which are the hypervariable regions of immunoglobulin heavy and light chains. *See, e.g.,* Kabat et al., Sequences of Proteins of Immunological Interest, 4th U. S. Department of Health and Human Services, National Institutes of Health (1987). Generally, antibodies comprise three heavy chain and three light chain CDRs or CDR regions in the variable region. CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. In certain embodiments, the CDRs regions are delineated using the Kabat system (Kabat, E. A., *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242).

As used herein, the term “single-chain variable fragment” or “scFv” is a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of an immunoglobulin covalently linked to form a $V_H::V_L$ heterodimer. The V_H and V_L are either joined directly or joined by a peptide-encoding linker (e.g., 10, 15, 20, 25 amino acids), which connects the N-terminus of the V_H with the C-terminus of the V_L , or the C-terminus of the V_H with the N-terminus of the V_L . The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility. Despite removal of the constant regions and the introduction of a linker, scFv proteins retain the specificity of the original immunoglobulin. Single chain Fv polypeptide antibodies can be expressed from a nucleic acid including V_H - and V_L -encoding sequences as described by Huston, et al. (Proc. Nat. Acad. Sci. USA, 85:5879-5883, 1988). *See, also*, U.S. Patent Nos. 5,091,513, 5,132,405

and 4,956,778; and U.S. Patent Publication Nos. 20050196754 and 20050196754.

Antagonistic scFvs having inhibitory activity have been described (see, e.g., Zhao et al.,
Hybridoma (Larchmt) 2008 27(6):455-51; Peter et al., J Cachexia Sarcopenia Muscle
2012 August 12; Shieh et al., J Immunol 2009 183(4):2277-85; Giomarelli et al., Thromb
5 Haemost 2007 97(6):955-63; Fife et al., J Clin Invest 2006 116(8):2252-61; Brocks et al.,
Immunotechnology 1997 3(3):173-84; Moosmayer et al., Ther Immunol 1995 2(10):31-
40). Agonistic scFvs having stimulatory activity have been described (see, e.g., Peter et
al., J Bio Chem 2003 25278(38):36740-7; Xie et al., Nat Biotech 1997 15(8):768-71;
Ledbetter et al., Crit Rev Immunol 1997 17(5-6):427-55; Ho et al., Biochim Biophys Acta
10 2003 1638(3):257-66).

As used herein, the term “affinity” is meant a measure of binding strength.
Affinity can depend on the closeness of stereochemical fit between antibody combining
sites and antigen determinants, on the size of the area of contact between them, and/or on
the distribution of charged and hydrophobic groups. As used herein, the term “affinity”
15 also includes “avidity”, which refers to the strength of the antigen-antibody bond after
formation of reversible complexes. Methods for calculating the affinity of an antibody
for an antigen are known in the art, including, but not limited to, various antigen-binding
experiments, e.g., functional assays (e.g., flow cytometry assay).

The term “chimeric antigen receptor” or “CAR” as used herein refers to a
20 molecule comprising an extracellular antigen-binding domain that is fused to an
intracellular signaling domain that is capable of activating or stimulating an
immunoresponsive cell, and a transmembrane domain. In certain embodiments, the
extracellular antigen-binding domain of a CAR comprises a scFv. The scFv can be
derived from fusing the variable heavy and light regions of an antibody. Alternatively or
25 additionally, the scFv may be derived from Fab’s (instead of from an antibody, e.g.,
obtained from Fab libraries). In certain embodiments, the scFv is fused to the
transmembrane domain and then to the intracellular signaling domain. In certain
embodiments, the CAR has a high binding affinity or avidity for the antigen.

As used herein, the term “nucleic acid molecules” include any nucleic acid
30 molecule that encodes a polypeptide of interest or a fragment thereof. Such nucleic acid
molecules need not be 100% homologous or identical with an endogenous nucleic acid
sequence, but may exhibit substantial identity. Polynucleotides having “substantial
identity” or “substantial homology” to an endogenous sequence are typically capable of
hybridizing with at least one strand of a double-stranded nucleic acid molecule. By

“hybridize” is meant a pair to form a double-stranded molecule between complementary polynucleotide sequences (e.g., a gene described herein), or portions thereof, under various conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) *Methods Enzymol.* 152:399; Kimmel, A. R. (1987) *Methods Enzymol.* 152:507).

5 For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, e.g., less than about 500 mM NaCl and 50 mM trisodium citrate, or less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least
10 about 35% formamide, e.g., at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C, at least about 37° C, or at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of
15 stringency are accomplished by combining these various conditions as needed. In certain embodiments, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In certain embodiments, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 µg/ml denatured salmon sperm DNA (ssDNA). In certain embodiments, hybridization will occur at 42° C
20 in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 µg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

 For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by
25 temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps can be less than about 30 mM NaCl and 3 mM trisodium citrate, e.g., less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, of at least about 42° C,
30 or of at least about 68° C. In certain embodiments, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In certain embodiments, wash steps will occur at 42° C. in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In certain embodiments, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those

skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Rogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

By “substantially identical” or “substantially homologous” is meant a polypeptide or nucleic acid molecule exhibiting at least about 50% homologous or identical to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). In certain embodiments, such a sequence is at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 99%, or at least about 100% homologous or identical to the sequence of the amino acid or nucleic acid used for comparison.

Sequence identity can be measured by using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

By “analog” is meant a structurally related polypeptide or nucleic acid molecule having the function of a reference polypeptide or nucleic acid molecule.

The term “ligand” as used herein refers to a molecule that binds to a receptor. In certain embodiments, the ligand binds to a receptor on another cell, allowing for cell-to-cell recognition and/or interaction.

The term “constitutive expression” or “constitutively expressed” as used herein refers to expression or expressed under all physiological conditions.

By “disease” is meant any condition, disease or disorder that damages or interferes with the normal function of a cell, tissue, or organ, e.g., neoplasia, and pathogen infection of cell.

By “effective amount” is meant an amount sufficient to have a therapeutic effect.

5 In certain embodiments, an “effective amount” is an amount sufficient to arrest, ameliorate, or inhibit the continued proliferation, growth, or metastasis (e.g., invasion, or migration) of a neoplasia.

By “enforcing tolerance” is meant preventing the activity of self-reactive cells or immunoresponsive cells that target transplanted organs or tissues.

10 By “endogenous” is meant a nucleic acid molecule or polypeptide that is normally expressed in a cell or tissue.

By “exogenous” is meant a nucleic acid molecule or polypeptide that is not endogenously present in a cell, or not present at a level sufficient to achieve the functional effects obtained when over-expressed. The term “exogenous” would therefore
15 encompass any recombinant nucleic acid molecule or polypeptide expressed in a cell, such as foreign, heterologous, and over-expressed nucleic acid molecules and polypeptides. By “exogenous” nucleic acid is meant a nucleic acid not present in a native wild-type cell; for example, an exogenous nucleic acid may vary from an endogenous counterpart by sequence, by position/location, or both. For clarity, an exogenous nucleic
20 acid may have the same or different sequence relative to its native endogenous counterpart; it may be introduced by genetic engineering into the cell itself or a progenitor thereof, and may optionally be linked to alternative control sequences, such as a non-native promoter or secretory sequence.

By a “heterologous nucleic acid molecule or polypeptide” is meant a nucleic acid
25 molecule (e.g., a cDNA, DNA or RNA molecule) or polypeptide that is not normally present in a cell or sample obtained from a cell. This nucleic acid may be from another organism, or it may be, for example, an mRNA molecule that is not normally expressed in a cell or sample.

By “immunoresponsive cell” is meant a cell that functions in an immune response
30 or a progenitor, or progeny thereof.

By “modulate” is meant positively or negatively alter. Exemplary modulations include a about 1%, about 2%, about 5%, about 10%, about 25%, about 50%, about 75%, or about 100% change.

By “increase” is meant to alter positively by at least about 5%. An alteration may be by about 5%, about 10%, about 25%, about 30%, about 50%, about 75%, about 100% or more.

By “reduce” is meant to alter negatively by at least about 5%. An alteration may be by about 5%, about 10%, about 25%, about 30%, about 50%, about 75%, or even by about 100%.

By “isolated cell” is meant a cell that is separated from the molecular and/or cellular components that naturally accompany the cell.

The terms “isolated,” “purified,” or “biologically pure” refer to material that is free to varying degrees from components which normally accompany it as found in its native state. “Isolate” denotes a degree of separation from original source or surroundings. “Purify” denotes a degree of separation that is higher than isolation. A “purified” or “biologically pure” protein is sufficiently free of other materials such that any impurities do not materially affect the biological properties of the protein or cause other adverse consequences. That is, a nucleic acid or peptide is purified if it is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Purity and homogeneity are typically determined using analytical chemistry techniques, for example, polyacrylamide gel electrophoresis or high performance liquid chromatography. The term “purified” can denote that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. For a protein that can be subjected to modifications, for example, phosphorylation or glycosylation, different modifications may give rise to different isolated proteins, which can be separately purified.

The term “antigen-binding domain” as used herein refers to a domain capable of specifically binding a particular antigenic determinant or set of antigenic determinants present on a cell.

“Linker”, as used herein, shall mean a functional group (e.g., chemical or polypeptide) that covalently attaches two or more polypeptides or nucleic acids so that they are connected to one another. As used herein, a “peptide linker” refers to one or more amino acids used to couple two proteins together (e.g., to couple V_H and V_L domains). In certain embodiments, the linker comprises a sequence set forth in GGGGSGGGGSGGGGS [SEQ ID NO: 31].

By “neoplasm” is meant a disease characterized by the pathological proliferation of a cell or tissue and its subsequent migration to or invasion of other tissues or organs. Neoplasia growth is typically uncontrolled and progressive, and occurs under conditions that would not elicit, or would cause cessation of, multiplication of normal cells.

- 5 Neoplasia can affect a variety of cell types, tissues, or organs, including but not limited to an organ selected from the group consisting of bladder, bone, brain, breast, cartilage, glia, esophagus, fallopian tube, gallbladder, heart, intestines, kidney, liver, lung, lymph node, nervous tissue, ovaries, pancreas, prostate, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, and
- 10 vagina, or a tissue or cell type thereof. Neoplasia include cancers, such as sarcomas, carcinomas, or plasmacytomas (malignant tumor of the plasma cells). In certain embodiments, the neoplasm is a solid tumor.

- Illustrative neoplasms for which the presently disclosed subject matter can be used include, but are not limited to leukemias (e.g., acute leukemia, acute lymphocytic
- 15 leukemia, acute myelocytic leukemia a, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, acute monocytic leukemia, acute erythroleukemia, chronic leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia), polycythemia vera, lymphoma (Hodgkin’s disease, non-Hodgkin’s disease), Waldenstrom’s macroglobulinemia, heavy chain disease, and solid tumors such as
- 20 sarcomas and carcinomas (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing’s tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma,
- 25 adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm’s tumor, cervical cancer, uterine cancer, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder
- 30 carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, schwannoma, meningioma, melanoma, neuroblastoma, and retinoblastoma).

By “receptor” is meant a polypeptide, or portion thereof, present on a cell membrane that selectively binds one or more ligand.

By “recognize” is meant selectively binds to a target. A T cell that recognizes a tumor can express a receptor (e.g., a TCR or CAR) that binds to a tumor antigen.

5 By “reference” or “control” is meant a standard of comparison. For example, the level of scFv-antigen binding by a cell expressing a CAR and an scFv may be compared to the level of scFv-antigen binding in a corresponding cell expressing CAR alone.

By “secreted” is meant a polypeptide that is released from a cell via the secretory pathway through the endoplasmic reticulum, Golgi apparatus, and as a vesicle that
10 transiently fuses at the cell plasma membrane, releasing the proteins outside of the cell.

By “signal sequence” or “leader sequence” is meant a peptide sequence (e.g., 5, 10, 15, 20, 25 or 30 amino acids) present at the N-terminus of newly synthesized proteins that directs their entry to the secretory pathway. Exemplary leader sequences include, but is not limited to, the IL-2 signal sequence: MYRMQLLSICALSLALVTNS [SEQ ID NO:
15 12] (human), MYSMQLASCVTLTLLVLLVNS [SEQ ID NO: 13] (mouse); the kappa leader sequence: METPAQLLFLLLLWLPDGTG [SEQ ID NO: 14] (human), METDTLLLWVLLLWVPGSTG [SEQ ID NO: 15] (mouse); the CD8 leader sequence: MALPVTALLPLALLHAARP [SEQ ID NO: 16] (human); the truncated human CD8 signal peptide: MALPVTALLPLALLHA [SEQ ID NO: 28] (human); the albumin
20 signal sequence: MKWVTFISLLFSSAYS [SEQ ID NO: 29] (human); and the prolactin signal sequence: MDSKGSSQKGSRLLLLVSNNLLCQGVVS [SEQ ID NO: 30] (human). By “soluble” is meant a polypeptide that is freely diffusible in an aqueous environment (e.g., not membrane bound).

By “specifically binds” is meant a polypeptide or fragment thereof that recognizes
25 and binds to a biological molecule of interest (e.g., a polypeptide), but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a presently disclosed polypeptide.

The term “tumor antigen” as used herein refers to an antigen (e.g., a polypeptide) that is uniquely or differentially expressed on a tumor cell compared to a normal or non-
30 IS neoplastic cell. In certain embodiments, a tumor antigen includes any polypeptide expressed by a tumor that is capable of activating or inducing an immune response via an antigen recognizing receptor (e.g., CD19, MUC-16) or capable of suppressing an immune response via receptor-ligand binding (e.g., CD47, PD-L1/L2, B7.1/2).

The terms “comprises”, “comprising”, and are intended to have the broad meaning ascribed to them in U.S. Patent Law and can mean “includes”, “including” and the like.

As used herein, “treatment” refers to clinical intervention in an attempt to alter the disease course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Therapeutic effects of treatment include, without limitation, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastases, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. By preventing progression of a disease or disorder, a treatment can prevent deterioration due to a disorder in an affected or diagnosed subject or a subject suspected of having the disorder, but also a treatment may prevent the onset of the disorder or a symptom of the disorder in a subject at risk for the disorder or suspected of having the disorder.

An “individual” or “subject” herein is a vertebrate, such as a human or non-human animal, for example, a mammal. Mammals include, but are not limited to, humans, primates, farm animals, sport animals, rodents and pets. Non-limiting examples of non-human animal subjects include rodents such as mice, rats, hamsters, and guinea pigs; rabbits; dogs; cats; sheep; pigs; goats; cattle; horses; and non-human primates such as apes and monkeys.

The term “immunocompromised” as used herein refers to a subject who has an immunodeficiency. The subject is very vulnerable to opportunistic infections, infections caused by organisms that usually do not cause disease in a person with a healthy immune system, but can affect people with a poorly functioning or suppressed immune system.

Other aspects of the presently disclosed subject matter are described in the following disclosure and are within the ambit of the presently disclosed subject matter.

2. HLA-Independent T Cell Receptor (HI-TCR)

The present disclosure provides an HI-TCR that binds to an antigen of interest in an HLA-independent manner. In certain non-limiting embodiments, binding of the antigen is capable of activating an immunoresponsive cell comprising the HI-TCR. In certain non-limiting embodiments, the HI-TCR comprises an antigen binding chain. In certain embodiments, the antigen binding chain comprises an extracellular antigen-binding domain. In certain embodiments, the extracellular antigen-binding domain is derived from a scFv, Fab, or antibody of murine, human or camelid (e.g., lama) origin. In certain embodiments, the antigen binding chain further comprises a constant domain.

2.1. *Antigens*

In certain embodiments, the HI-TCR binds to a tumor antigen. Any tumor antigen (antigenic peptide) can be used in the tumor-related embodiments described herein.

Sources of antigen include, but are not limited to, cancer proteins. The antigen can be expressed as a peptide or as an intact protein or portion thereof. The intact protein or a portion thereof can be native or mutagenized. Non-limiting examples of tumor antigens include carbonic anhydrase IX (CAIX), carcinoembryonic antigen (CEA), CD8, CD7, CD10, CD19, CD20, CD22, CD30, CD33, CLL1, CD34, CD38, CD41, CD44, CD49f, CD56, CD74, CD133, CD138, CD123, CD44V6, an antigen of a cytomegalovirus (CMV) infected cell (e.g., a cell surface antigen), epithelial glycoprotein-2 (EGP-2), epithelial glycoprotein-40 (EGP-40), epithelial cell adhesion molecule (EpCAM), receptor tyrosine-protein kinases erb-B2,3,4 (erb-B2,3,4), folate-binding protein (FBP), fetal acetylcholine receptor (AChR), folate receptor- α , Ganglioside G2 (GD2), Ganglioside G3 (GD3), human Epidermal Growth Factor Receptor 2 (HER-2), human telomerase reverse transcriptase (hTERT), Interleukin-13 receptor subunit alpha-2 (IL-13R α 2), κ -light chain, kinase insert domain receptor (KDR), Lewis Y (LeY), L1 cell adhesion molecule (L1CAM), melanoma antigen family A, 1 (MAGE-A1), Mucin 16 (MUC16), Mucin 1 (MUC1), Mesothelin (MSLN), ERBB2, MAGEA3, p53, MART1, GP100, Proteinase3 (PR1), Tyrosinase, Survivin, hTERT, EphA2, NKG2D ligands, cancer-testis antigen NY-ESO-1, oncofetal antigen (h5T4), prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA), ROR1, tumor-associated glycoprotein 72 (TAG-72), vascular endothelial growth factor R2 (VEGF-R2), and Wilms tumor protein (WT-1), BCMA, NKCS1, EGF1R, EGFR-VIII, CD99, CD70, ADGRE2, CCR1, LILRB2, LILRB4, PRAME and ERBB.

In certain embodiments, the HI-TCR binds to CD19. In certain embodiments, the HI-TCR binds to a human CD19 polypeptide. In certain embodiments, the human CD19 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 11.

PEEPLVVKVEEGDNAVLQCLKGTSDGPTQQLTWSRESPLKPFLLKSLGLPGLGIHMRPLAIWLFI FNVSQQM
GGFYLCQPGPPSEKAWQPGWTVNVEGSGELFRWNVSDLGGLGCGLKNSSEGPPSSPGKLMSPKLYVWAKDR
30 PEIWEGEPPCLPPRDSL NQSL SQDLTMAPGSTLWLSCGVPPDSVSRGPLSWTHVHPKGPKSLLSLELKDDRP
ARDMWVMTGLLLPRATAQDAGKYCHRGNTMSFHLEITARPVLWHWLLRTGGWK [SEQ ID NO:
11].

In certain embodiments, the HI-TCR binds to the extracellular domain of a CD19 protein.

In certain embodiments, the HI-TCR binds to a pathogen antigen, e.g., for use in treating and/or preventing a pathogen infection or other infectious disease, for example, in an immunocompromised subject. Non-limiting examples of pathogen includes a virus, bacteria, fungi, parasite and protozoa capable of causing disease.

5 Non-limiting examples of viruses include, *Retroviridae* (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HDTV-III, LAVE or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; *Picornaviridae* (e.g. polio viruses, hepatitis A virus; enteroviruses, human Cocksackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g. strains that cause gastroenteritis); *Togaviridae* (e.g. equine encephalitis viruses, rubella viruses); *Flaviridae* (e.g. dengue viruses, encephalitis
10 viruses, yellow fever viruses); *Coronaviridae* (e.g. coronaviruses); *Rhabdoviridae* (e.g. vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g. ebola viruses); *Paramyxoviridae* (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g. influenza viruses); *Bungaviridae* (e.g. Hantaan
15 viruses, bunga viruses, phleboviruses and Naira viruses); *Arena viridae* (hemorrhagic fever viruses); *Reoviridae* (e.g. reoviruses, orbiviruses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV),
20 herpes virus; *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (e.g. African swine fever virus); and unclassified viruses (e.g. the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1 =internally transmitted; class 2 =parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

25 Non-limiting examples of bacteria include *Pasteurella*, *Staphylococci*, *Streptococcus*, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to, *Helicobacter pylori*, *Borelia burgdorferi*, *Legionella pneumophila*, *Mycobacteria* sps (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. goodii*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium*

diphtheriae, *corynebacterium sp.*, *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*,
Clostridium tetani, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella*
multocida, *Bacteroides sp.*, *Fusobacterium nucleatum*, *Streptobacillus moniliformis*,
Treponema pallidum, *Treponema pertenue*, *Leptospira*, *Rickettsia*, and *Actinomyces*
5 *israeli*.

In certain embodiments, the pathogen antigen is a viral antigen present in Cytomegalovirus (CMV), a viral antigen present in Epstein Barr Virus (EBV), a viral antigen present in Human Immunodeficiency Virus (HIV), or a viral antigen present in influenza virus.

10 2.2. Extracellular Antigen-Binding Domain

In certain embodiments, the antigen binding chain comprises an extracellular antigen-binding domain. In certain embodiments, the extracellular antigen-binding domain specifically binds to an antigen, e.g., a tumor antigen or a pathogen antigen, e.g., those disclosed in Section 2.1. In certain embodiments, the extracellular antigen-binding
15 domain is capable of dimerizing with another extracellular antigen-binding domain (e.g., forming a fragment variable (Fv)), wherein the dimerized antigen-binding domains (e.g., an Fv) specifically bind to an antigen, e.g., a tumor antigen or a pathogen antigen.

In certain embodiments, the extracellular antigen-binding domain comprises a ligand for a cell-surface receptor. In certain embodiments, the extracellular antigen-
20 binding domain comprises a receptor for a cell surface ligand.

In certain embodiments, the extracellular antigen-binding domain specifically binds to an antigen, e.g., a tumor antigen or a pathogen antigen. In certain embodiments, the antigen binding chain is capable of forming a dimer with another antigen binding chain. In certain embodiments, the HI-TCR comprises a heterodimer comprising two
25 different antigen binding chains. In certain embodiments, the HI-TCR comprises a homodimer comprising two identical antigen binding chains. In certain embodiments, the antigen binding chains dimerize through one or more disulfide-links. In certain embodiments, the antigen binding chain is capable of forming a trimer or oligomer with one or more identical or different antigen binding chains. In certain embodiments, the
30 extracellular antigen-binding domain is capable of dimerizing with another extracellular antigen-binding domain (e.g., forming a fragment variable (Fv)), wherein the dimerized antigen-binding domains (e.g., a Fv) specifically bind to an antigen, e.g., a tumor antigen or a pathogen antigen.

In certain non-limiting embodiments, the extracellular antigen-binding domain of the HI-TCR (for example, an Fv or an analog thereof) binds to an antigen with a dissociation constant (K_d) of about 2×10^{-7} M or less. In certain embodiments, the K_d is about 2×10^{-7} M or less, about 1×10^{-7} M or less, about 9×10^{-8} M or less, about 1×10^{-8} M or less, about 9×10^{-9} M or less, about 5×10^{-9} M or less, about 4×10^{-9} M or less, about 3×10^{-9} M or less, about 2×10^{-9} M or less, or about 1×10^{-9} M or less. In certain non-limiting embodiments, the K_d is about 3×10^{-9} M or less. In certain non-limiting embodiments, the K_d is from about 1×10^{-9} M to about 3×10^{-7} M. In certain non-limiting embodiments, the K_d is from about 1.5×10^{-9} M to about 3×10^{-7} M. In certain non-limiting embodiments, the K_d is from about 1.5×10^{-9} M to about 2.7×10^{-7} M.

Binding of the extracellular antigen-binding domain (for example, a Fv or an analog thereof) can be confirmed by, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), FACS analysis, bioassay (*e.g.*, growth inhibition), or Western Blot assay. Each of these assays generally detect the presence of protein-antibody complexes of particular interest by employing a labeled reagent (*e.g.*, an antibody, or an Fv) specific for the complex of interest. For example, the Fv can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography. In certain embodiments, the extracellular antigen-binding domain is labeled with a fluorescent marker. Non-limiting examples of fluorescent markers include green fluorescent protein (GFP), blue fluorescent protein (*e.g.*, EBFP, EBFP2, Azurite, and mKalamal), cyan fluorescent protein (*e.g.*, ECFP, Cerulean, and CyPet), and yellow fluorescent protein (*e.g.*, YFP, Citrine, Venus, and YPet).

In certain embodiments, the extracellular antigen-binding domain comprises an antigen binding portion of a TCR.

In certain embodiments, the extracellular antigen-binding domain comprises an antigen binding portion of an antibody or a fragment thereof. In certain embodiments, the extracellular antigen-binding domain comprises a heavy chain variable region (V_H) and/or a light chain variable region (V_L) of an antibody. In certain embodiments, the extracellular antigen-binding domain comprises a single-chain variable fragment (scFv). In certain embodiments, the extracellular antigen-binding domain comprises a heavy

chain-only antibodies (VHH). In certain embodiments, the extracellular antigen-binding domain comprises a Fab, which is optionally crosslinked. In certain embodiments, the extracellular antigen-binding domain comprises a F(ab)₂. In certain embodiments, any of the foregoing molecules can be comprised in a fusion protein with a heterologous
5 sequence to form the extracellular antigen-binding domain.

In certain embodiments, the extracellular antigen-binding domain comprises a heavy chain variable region (V_H) and/or a light chain variable region (V_L) of an antibody, wherein the V_H or the V_L is capable of dimerizing with another extracellular antigen-binding domain comprising a V_L or a V_H (e.g., forming a fragment variable (Fv)). In
10 certain embodiments, the Fv is a human Fv. In certain embodiments, the Fv is a humanized Fv. In certain embodiments, the Fv is a murine Fv. In certain embodiments, the Fv is identified by screening a Fv phage library with an antigen-Fc fusion protein.

Additional extracellular antigen-binding domains targeting an interested antigen can be obtained by sequencing an existing scFv or a Fab region of an existing antibody
15 targeting the same antigen.

In certain embodiments, the dimerized extracellular antigen-binding domain of a presently disclosed HI-TCR is a murine Fv. In certain embodiments, the dimerized extracellular antigen-binding domain is an Fv that binds to a human CD19 polypeptide. In certain embodiments, the extracellular antigen-binding domain is an Fv, which
20 comprises the amino acid sequence of SEQ ID NO: 9 and specifically binds to a human CD19 polypeptide (e.g., a human CD19 polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 11). In certain embodiments, the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 9 is set forth in SEQ ID NO: 10.

In certain embodiments, the Fv comprises a heavy chain variable region (V_H) comprising the amino acid sequence set forth in SEQ ID NO: 7. In certain embodiments, the Fv comprises a light chain variable region (V_L) comprising the amino acid sequence set forth in SEQ ID NO: 8. In certain embodiments, the Fv comprises V_H comprising the amino acid sequence set forth in SEQ ID NO: 7 and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 8. In certain embodiments, the extracellular antigen-binding domain comprises a V_H comprising an amino acid sequence that is at least about
30 80% (e.g., at least about 85%, at least about 90%, or at least about 95%) homologous or identical to SEQ ID NO: 7. For example, the extracellular antigen-binding domain comprises a V_H comprising an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about

89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% homologous or identical to SEQ ID NO: 7. In certain embodiments, the extracellular antigen-binding domain comprises a V_H comprising the amino sequence set forth in SEQ ID NO: 7. In certain embodiments, the extracellular antigen-binding domain comprises a V_L comprising an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) homologous to SEQ ID NO: 8. For example, the extracellular antigen-binding domain comprises a V_L comprising an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% homologous or identical to SEQ ID NO: 8. In certain embodiments, the extracellular antigen-binding domain comprises a V_L comprising the amino acid sequence set forth in SEQ ID NO: 8. In certain embodiments, the extracellular antigen-binding domain comprises a V_H comprising an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) homologous to SEQ ID NO: 7, and a V_L comprising an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) homologous or identical to SEQ ID NO: 8. In certain embodiments, the extracellular antigen-binding domain comprises a V_H comprising the amino acid sequence set forth in SEQ ID NO: 7 and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 8.

In certain embodiments, the Fv comprises a heavy chain variable region (V_H) comprising the amino acid sequence set forth in SEQ ID NO: 44. In certain embodiments, the Fv comprises a light chain variable region (V_L) comprising the amino acid sequence set forth in SEQ ID NO: 45. In certain embodiments, the Fv comprises V_H comprising the amino acid sequence set forth in SEQ ID NO: 44 and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 45. In certain embodiments, the extracellular antigen-binding domain comprises a V_H comprising an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) homologous or identical to SEQ ID NO: 44. For example, the extracellular antigen-binding domain comprises a V_H comprising an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% homologous or identical to SEQ

ID NO: 44. In certain embodiments, the extracellular antigen-binding domain comprises a V_H comprising the amino sequence set forth in SEQ ID NO: 44. In certain embodiments, the extracellular antigen-binding domain comprises a V_L comprising an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) homologous to SEQ ID NO: 45. For example, the extracellular antigen-binding domain comprises a V_L comprising an amino acid sequence that is at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% homologous or identical to SEQ ID NO: 45. In certain embodiments, the extracellular antigen-binding domain comprises a V_L comprising the amino acid sequence set forth in SEQ ID NO: 45. In certain embodiments, the extracellular antigen-binding domain comprises a V_H comprising an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) homologous to SEQ ID NO: 44, and a V_L comprising an amino acid sequence that is at least about 80% (*e.g.*, at least about 85%, at least about 90%, or at least about 95%) homologous or identical to SEQ ID NO: 45. In certain embodiments, the extracellular antigen-binding domain comprises a V_H comprising the amino acid sequence set forth in SEQ ID NO: 44 and a V_L comprising the amino acid sequence set forth in SEQ ID NO: 45.

In certain embodiments, the extracellular antigen-binding domain comprises a V_H CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, or a conservative modification thereof, a V_H CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 2 or a conservative modification thereof, and a V_H CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 3, a conservative modification thereof. In certain embodiments, the extracellular antigen-binding domain comprises a V_H CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, a V_H CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 2, and a V_H CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 3. In certain embodiments, the extracellular antigen-binding domain comprises a V_L CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 4 or a conservative modification thereof, a V_L CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5 or a conservative modification thereof, and a V_L CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6 or a conservative modification thereof. In certain embodiments, the extracellular antigen-binding domain comprises a V_L CDR1 comprising the amino acid

sequence set forth in SEQ ID NO: 4, a V_L CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and a V_L CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6. In certain embodiments, the extracellular antigen-binding domain comprises a V_H CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1 or a conservative modification thereof, a V_H CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 2 or a conservative modification thereof, a V_H CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 3, a conservative modification thereof, a V_L CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 4 or a conservative modification thereof, a V_L CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5 or a conservative modification thereof, and a V_L CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6 or a conservative modification thereof. In certain embodiments, the extracellular antigen-binding domain comprises a V_H CDR1 comprising amino acids having the sequence set forth in SEQ ID NO: 1, a V_H CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 2, a V_H CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 3, a V_L CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 4, a V_L CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5 and a V_L CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6.

Table 1

		anti-human CD19 scFv (SJ25C1)		
CDRs		1	2	3
V _H	a.a.	GYAFSS [SEQ ID NO: 1]	YPGDGD [SEQ ID NO: 2]	KTISSVDF [SEQ ID NO: 3]
V _L	a.a.	KASQNVGTNVA [SEQ ID NO: 4]	SATYRN [SEQ ID NO: 5]	QQYNRYPT [SEQ ID NO: 6]
Full V _H		EVKLQQSGAE LVRPGSSVKI SCKASGYAFS SYWMNWVKQR PGQGLEWIGQ IYPGDGDTNY NGKFKGQATL TADKSSSTAY MQLSGLTSED SAVYFCARKT ISSVDFYFD YWGQGT TVTV SS [SEQ ID NO: 7]		
Full V _L		DIELTQSPKF MSTSVGDRVS VTCKASQNVG TNVAWYQQKP GQSPKPLIYS ATYRNSGVPD RFTGSGSGTD FTLTITNVQS KDLADYFCQQ YNRYPTSGG GTKLEIKR [SEQ ID NO: 8]		
scFv		MALPVTALLL PLALLLHAEV KLQQSGAELV RPGSSVKISC KASGYAFSSY WMNWVKQRPQ QGLEWIGQIY PGDGDNTNYG KFKGQATLTA DKSSSTAYMQ LSGLTSEDSA VYFCARKTIS SVDFYFDYW GQGT TVTVSS GGGGSGGGGS GGGGSDIELT QSPKFMSTSV GDRVSVTCKA SQNVGTNVAW YQQKPGQSPK PLIYSATYRN SGVPDRETGS GSGTDFTLTI TNVQSKDLAD YFCQQYNRYP		

	YTSGGGKLE IKR [SEQ ID NO: 9]
DNA	ATGGCTCTCCAGTGACTGCCCTACTGCTTCCCCTAGCGCTTCTCCTGCATGCAGAGG TGAAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCTGGGTCTCAGTGAAGATTTC CTGCAAGGCTTCTGGCTATGCATTAGTAGCTACTGGATGAACTGGGTGAAGCAGAGG CCTGGACAGGGTCTTGAGTGGATTGGACAGATTTATCCTGGAGATGGTGATACTAACT ACAATGGAAAGTTCAAGGGTCAAGCCACACTGACTGCAGACAAATCCTCCAGCACAGC CTACATGCAGCTCAGCGGCCTAACATCTGAGGACTCTGCGGTCTATTCTGTGCAAGA AAGACCATTAGTTCGGTAGTAGATTTCTACTTTGACTACTGGGGCCAAGGGACCACGG TCACCGTCTCCTCAGGTGGAGGTGGATCAGGTGGAGGTGGATCTGGTGGAGGTGGATC TGACATTGAGCTCACCCAGTCTCCAAAATTCATGTCCACATCAGTAGGAGACAGGGTC AGCGTCACCTGCAAGGCCAGTCAGAATGTGGGTACTAATGTAGCCTGGTATCAACAGA AACCAGGACAATCTCCTAAACCACTGATTACTCGGCAACCTACCGGAACAGTGGAGT CCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCACTAAC GTGCAGTCTAAAGACTTGGCAGACTATTTCTGTCAACAATATAACAGGTATCCGTACA CGTCCGGAGGGGGGACCAAGCTGGAGATCAAACGG [SEQ ID NO: 10]

As used herein, the term “a conservative sequence modification” refers to an amino acid modification that does not significantly affect or alter the binding characteristics of the presently disclosed HI-TCR comprising the amino acid sequence.

- 5 Conservative modifications can include amino acid substitutions, additions and deletions. Modifications can be introduced into the Fv of the presently disclosed HI-TCR by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Amino acids can be classified into groups according to their physicochemical properties such as charge and polarity. Conservative amino acid
- 10 substitutions are ones in which the amino acid residue is replaced with an amino acid within the same group. For example, amino acids can be classified by charge: positively-charged amino acids include lysine, arginine, histidine, negatively-charged amino acids include aspartic acid, glutamic acid, neutral charge amino acids include alanine, asparagine, cysteine, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine,
- 15 proline, serine, threonine, tryptophan, tyrosine, and valine. In addition, amino acids can be classified by polarity: polar amino acids include arginine (basic polar), asparagine, aspartic acid (acidic polar), glutamic acid (acidic polar), glutamine, histidine (basic polar), lysine (basic polar), serine, threonine, and tyrosine; non-polar amino acids include alanine, cysteine, glycine, isoleucine, leucine, methionine, phenylalanine, proline,
- 20 tryptophan, and valine. Thus, one or more amino acid residues within a CDR region can be replaced with other amino acid residues from the same group and the altered antibody can be tested for retained function (*i.e.*, the functions set forth in (c) through (l) above) using the functional assays described herein. In certain embodiments, no more than one, no more than two, no more than three, no more than four, no more than five residues
- 25 within a specified sequence or a CDR region are altered.

The V_H and/or V_L amino acid sequences having at least about 80%, at least about 85%, at least about 90%, or at least about 95% (*e.g.*, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%) homology to a specific sequence (*e.g.*, SEQ ID NOs: 7, and 8) may contain substitutions (*e.g.*, conservative substitutions), insertions, or deletions relative to the specified sequence(s), but retain the ability to bind to a target antigen (*e.g.*, CD19). In certain embodiments, a total of 1 to 10 amino acids are substituted, inserted and/or deleted in a specific sequence (*e.g.*, SEQ ID NOs: 7, and 8). In certain embodiments, substitutions, insertions, or deletions occur in regions outside the CDRs (*e.g.*, in the FRs) of the extracellular antigen-binding domain. In certain embodiments, the extracellular antigen-binding domain comprises V_H and/or V_L sequence selected from the group consisting of SEQ ID NOs: 7, and 8, including post-translational modifications of that sequence (SEQ ID NOs: 7 and 8).

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

The percent homology between two amino acid sequences can be determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.*, 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent homology between two amino acid sequences can be determined using the Needleman and Wunsch (*J. Mol. Biol.* 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

Additionally or alternatively, the amino acids sequences of the presently disclosed subject matter can further be used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be

performed using the XBLAST program (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the specified sequences (e.g., heavy and light chain variable region sequences of scFv m903, m904, m905, m906, and m900) disclosed herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

10 2.3. Constant Domain

In certain embodiments, the antigen binding chain further comprises a constant domain. In certain embodiments, the constant domain comprises a hinge/spacer region and a transmembrane domain. In certain embodiments, the constant domain is capable of forming a homodimer or a heterodimer with another constant domain. In certain
15 embodiments, the constant domain dimerizes through one or more disulfide-links. In certain embodiments, the antigen binding chain is capable of forming a trimer or oligomer with one or more identical or different constant domains.

In certain non-limiting embodiments, the constant domain comprises a T cell receptor constant region, e.g., T cell receptor alpha constant region (TRAC), T cell
20 receptor beta constant region (TRBC, e.g., TRBC1 or TRBC2), T cell receptor gamma constant region (TRGC, e.g., TRGC1 or TRGC2), T cell receptor delta constant region (TRDC) or any variants or functional fragments thereof.

In certain embodiments, the constant domain of a presently disclosed HI-TCR comprises a native or modified TRAC peptide. In certain embodiments, the TRAC polypeptide comprises an amino acid sequence that is at least about 80%, at least about
25 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or at least about 100% homologous or identical to the amino acid sequence set forth in SEQ ID NO:38, which is provided below), or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative
30 amino acid substitutions.

IQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKSDVYITDKTVLDMRSMDFKSNSAVAWSNKSDFAC
ANAFNNSIIPEDTFFPSPESCDVKLVEKSFETDTNLFQNLVIGFRILLKLVAGFNLLMTLRWSS
[SEQ ID NO:38]

In certain embodiments, the TRAC polypeptide has an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or at least about 100% homologous or identical to the amino acid sequence encoded by a transcript expressed by the gene of NCBI Genbank ID: 28755, NG_001332.3, range 925603 to 930229 (SEQ ID NO:29, which is provided below), or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

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10 1  atatccagaa cctgaccct gccgtgtacc agctgagaga ctctaaatcc agtgacaagt
    61  ctgtctgcct attcaccgat tttgattctc aaacaaatgt gtcacaaagt aaggattctg
    121 atgtgtatat cacagacaaa actgtgctag acatgaggtc tatggacttc aagagcaaca
    181 gtgtgtgggc ctggagcaac aaatctgact ttgcatgtgc aaacgccttc aacaacagca
    241 ttattccaga agacaccttc ttccccagcc caggtaaggg cagctttggt gccttcgcag
    301 gctgtttcct tgcttcagga atggccaggt tctgcccaga gctctgggtc atgatgtcta
15 361 aaactcctct gattgggtgg ctggccctta tccattgccca ccaaaacccct ctttttacta
    421 agaaacagtg agccttgctc tggcagtgcca gagaatgaca cgggaaaaaa gcagatgaag
    481 agaaggtggc aggagagggc acgtggccca gcctcagtct ctccaactga gttcctgcct
    541 gcctgccttt gctcagactg tttgcccctt actgctcttc taggcctcat tctaagcccc
    601 ttctccaagt tgctctcctt tatttctccc tgtctgccaa aaaatctttc ccagctcact
20 661 aagtcagtct cagcagtc ctcattaacc caccaatcac tgattgtgcc ggcacatgaa
    721 tgcaccaggt gttgaagtgg aggaattaaa aagtcagatg aggggtgtgc ccagaggaa
    781 caccattcta gttgggggag cccatctgtc agctgggaaa agtccaaata acttcagatt
    841 ggaatgtgtt ttaactcagg gttgagaaaa cagctacctt caggacaaaa gtcagggaag
    901 ggctctctga agaaatgcta cttgaagata ccagccctac caagggcagg gagaggaccc
25 961 tatagaggcc tgggacagga gctcaatgag aaaggagaag agcagcaggg atgagttgaa
    1021 tgaaggagggc agggccgggt cacaggccct tctaggccat gagagggtag acagtattct
    1081 aaggacgcca gaaagctgtt gatcggcttc aagcagggga gggacaccta atttgctttt
    1141 cttttttttt tttttttttt tttttttttt tgagatggag ttttgcctct gttgcccagg
    1201 ctggagtgca atggtgcatc ttggctcact gcaacctccg cctcccaggt tcaagtgatt
30 1261 ctctgcctc agcctcccga gtagctgaga ttacaggcac ccgccaccat gcctggctaa
    1321 ttttttgtat ttttagtaga gacagggttt cactatgttg gccagggttg tctcgaactc
    1381 ctgacctcag gtgatccacc cgcttcagcc tcccacagtg ctgggattac aggcgtgagc
    1441 caccacaccc ggctgctttt tcttaagat caatctgagt gctgtacgga gagtgggttg
    1501 taagccaaga gtagaagcag aaaggagca gttgcagcag agagatgatg gaggcctggg
35 1561 cagggtgggt gcaggaggtt aaccaacacc attcaggttt caaaggtaga accatgcagg
    1621 gatgagaaag caaagagggg atcaaggaag gcagctggat tttggcctga gcagctgagt
    1681 caatgatagt gccgtttact aagaagaaac caaggaaaaa atttgggggtg cagggatcaa
    1741 aacttttttg aacatatgaa agtacgtgtt tatactcttt atggcccttg tcaatatgta
    1801 tgctcgctg cctccattgg actctagaat gaagccaggc aagagcaggg tctatgtgtg
40 1861 atggcacatg tggccagggt catgcaacat gtactttgta caaacagtgt atattgagta
    1921 aatagaaatg gtgtccagga gccgaggtat cggctcctgc agggccaggg gctctcccta
    1981 gcagggtgctc atatgctgta agttccctcc agatctctcc acaaggaggc atggaaaggc
    2041 tgtagttggt cacctgccc aagaactagga ggtctggggg gggagagtca gcctgctctg
    2101 gatgctgaaa gaatgtctgt ttttcctttt agaaagttcc tgtgatgtca agctggtctga
45 2161 gaaaagcttt gaaacaggta agacaggggt ctagcctggg tttgcacagg attgcggaag
    2221 tgatgaaccc gcaataaccc tgcctggatg agggagtggg aagaaattag tagatgtggg
    2281 aatgaatgat gaggaatgga aacagcggtt caagacctgc ccagagctgg gtgggggtctc
    2341 tcttgaatcc ctctcaccat ctctgacttt ccattctaag cactttgagg atgagtttct
    2401 agcttcaata gaccaaggac tctctcctag gcctctgtat tcctttcaac agctccactg
50 2461 tcaagagagc cagagagagc tctgtgtggg cccagctgtg aaatttctga gtcccttagg
    2521 gatagcccta aacgaaccag atcatcctga ggacagccaa gaggttttgc cttctttcaa
    2581 gacaagcaac agtactcaca taggctgtgg gcaatgggtc tgtctctcaa gaatcccctg
    2641 ccaactctca caccaccctt gggcccatat tcatttccat ttgagttgtt cttattgagt
    2701 catccttctt gtggtagcgg aactcactaa ggggcccatc tggaccagag gtattgtgat

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2761 gataaattct gagcacctac cccatcccca gaagggtcca gaaataaaat aagagccaag
2821 tctagtcggt gtttcctgtc ttgaaacaca atactgttg ccttgaaga atgcacagaa
2881 tctgtttgta aggggatatg cacagaagct gcaaggga ggaggtgcag gagctgcagg
2941 cctcccccac ccagcctgct ctgccttggg gaaaaccgtg ggtgtgtcct gcaggccatg
5 3001 caggcctggg acatgcaagc ccataaccgc tgtggcctct tggttttaca gatacgaacc
3061 taaactttca aaacctgtca gtgattgggt tccgaatcct cctcctgaaa gtggccgggt
3121 ttaatctgct catgacgctg cggtgtggt ccagctgagg tgaggggctt tgaagctggg
3181 agtggggttt agggacgcgg gtctctgggt gcatcctaag ctctgagagc aaacctccct
3241 gcagggtcct gcttttaagt ccaaagcctg agcccaccaa actctcctac ttcttccgtg
10 3301 tacaaattcc tctgtgcaa taataatggc ctgaaacgct gtaaaatata ctcatttcag
3361 ccgcctcagt tgcaattctc ccctatgagg taggaagaac agttgtttag aaacgaagaa
3421 actgaggccc cacagctaag gactggagga agagagacac ttgtgtacac cacatgcctt
3481 gtgtgtgact tctctaccg tgtaacctcc tcatgtctc tctcccagc acggctctct
3541 tagctcagta gaaagaagac attacactca tattacacc caatcctggc tagagtctcc
15 3601 gcaccctcct ccccagggt cccagtcgt cttgctgaca actgcatcct gttccatcac
3661 catcaaaaaa aaactccagg ctgggtgcgg gggctcacac ctgtaatccc agcacttttg
3721 gaggcagagg caggaggagc acaggagctg gagaccagcc tgggcaacac agggagaccc
3781 cgcctctaca aaaagtga aaattaacca ggtgtgtgtc tgcacacctg tagtcccagc
3841 tacttaagag gctgagatgg gaggatcgct tgagccctgg aatgttgagg ctacaatgag
20 3901 ctgtgattgc gtcactgcac tccagcctgg aagacaaagc aagatcctgt ctcaaataat
3961 aaaaaaata agaactccag ggtacatttg ctctagaac tctaccacat agccccaac
4021 agagccatca ccatcacatc cctaacagtc ctgggtcttc ctcagtgtcc agcctgactt
4081 ctgttcttcc tcattccaga tctgcaagat tgtaagacag cctgtgtctc ctgcgtcctt
4141 cctctgcatt gccctcttc tccctctcca aacagaggga actctcctac cccaaggag
25 4201 gtgaaagctg ctaccacctc tgtgcccccc cggcaatgcc accaactgga tctaccgga
4261 atttatgatt aagattgctg aagagctgcc aaacactgct gccacccct ctgttccctt
4321 attgtgtcct gtcactgcct gacattcacg gcagaggcaa ggctgtgca gcctcccctg
4381 gctgtgcaca ttccctcctg ctcccagag actgcctccg ccatcccaca gatgatggat
4441 cttcagtggg ttctcttggg ctctaggtcc tgcagaatgt tgtgaggggt ttattttttt
30 4501 ttaatagtgt tcataaagaa atacatagta ttcttcttct caagacgtgg ggggaaatta
4561 tctcattatc gaggccctgc tatgtgtgt atctgggcgt gttgtatgtc ctgctgccga
4621 tgccttc [SEQ ID NO:29]

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In accordance with the presently disclosed subject matter, an “TRAC nucleic acid molecule” refers to a polynucleotide encoding an TRAC polypeptide.

35 In certain embodiments, the constant domain of a presently disclosed HI-TCR comprises a native or modified TRBC peptide. In certain embodiments, the constant domain of a presently disclosed HI-TCR comprises a native or modified TRBC2 peptide. In certain embodiments, the TRBC2 polypeptide comprises an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or at least about 100% homologous or identical to the amino acid sequence set forth in SEQ ID NO:39, which is provided below, or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

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DLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYDPDHVELSWVNGKEVHSGVSTDPQPLKEQPALNDSR
45 YCLSSRLRVSATFWQNPFRNHFRQVQFYGLSENDEWTDRAKPVTVQIVSAEAWGRADCGFTSESYQQGVLSA
TILYEILLGKATLYAVLVLSALVLMAMVKRKDSRG [SEQ ID NO:39]

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In certain embodiments, the constant domain of a presently disclosed HI-TCR comprises a native or modified TRBC1 peptide. In certain embodiments, the TRBC1 polypeptide comprises an amino acid sequence that is at least about 80%, at least about

85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or at least about 100% homologous or identical to the amino acid sequence set forth in SEQ ID NO:40, which is provided below, or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

```
DLNKVFPPEV AVFEPSEAEI SHTQKATLVC LATGFFPDHV ELSWWVNGKE
VHSGVSTDPQ PLKEQPALND SRYCLSSRLR VSATFWQNPR NHFRCQVQFY
GLSENDEWTQ DRAKPVQTIV SAEAWGRADC GFTSVSYQQG VLSATILYEI
LLGKATLYAV LVSAVLMMAM VKRKDF [SEQ ID NO:40]
```

In certain embodiments, the TRBC polypeptide has an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or at least about 100% homologous or identical to the amino acid sequence encoded by a transcript expressed by a gene of NCBI Genbank ID: 28639, NG_001333.2, range 645749 to 647196 (TRBC1, SEQ ID NO: 30), NCBI Genbank ID: 28638, NG_001333.2 range 655095 to 656583 (TRBC2, SEQ ID NO:31) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

```
1 aggacctgaa caaggtgttc ccaccgagg tcgctgtgtt tgagccatca gaagcagaga
61 tctccacac ccaaaaggcc acactggtgt gcctggccac aggtctcttc cccgaccacg
121 tggagctgag ctggtgggtg aatgggaagg aggtgcacag tggggtcagc acagaccgcg
181 agccctcaa ggagcagccc gccctcaatg actccagata ctgcctgagc agcgcctga
241 gggctctggc caccttctgg cagaaccccc gcaaccactt ccgctgtcaa gtccagttct
301 acgggctctc ggagaatgac gagtggaccc aggatagggc caaacccgtc acccagatcg
361 tcagcgccga ggcctggggt agagcaggtg agtggggcct ggggagatgc ctggaggaga
421 ttaggtgaga ccagctacca gggaaaatgg aaagatccag gtagcagaca agactagatc
481 caaaaagaaa ggaaccagcg cacaccatga aggagaattg ggcacctgtg gttcattctt
541 ctcccagatt ctcagcccaa cagagccaag cagctgggtc ccctttctat gtggcctgtg
601 taactctcat ctgggtgggt ccccccaccc ccctcagtgc tgccacatgc catggattgc
661 aaggacaatg tggtgacat ctgcatggca gaagaaagga ggtgctgggc tgtcagagga
721 agctggtctg ggcctgggag tctgtgcaa ctgcaaatct gactttactt ttaattgcct
781 atgaaaataa ggtctctcat ttattttcct ctccctgctt tctttcagac tgtggcttta
841 cctcgggtaa gtaagccctt ccttttcctc tcctctctc atggttcttg acctagaacc
901 aaggcatgaa gaactcacag acactggagg gtggagggtg ggagagacca gagctacctg
961 tgcacaggta cccacctgtc ctctctcctg gccaacagtg tcctaccagc aaggggtcct
1021 gtctgccacc atcctctatg agatcctgct aggggaaggcc accctgtatg ctgtgctggt
1081 cagcgccctt gtgttgatgg ccatggtgaag caggagggca ggtggggcc agcaggctgg
1141 aggtgacaca ctgacaccaa gcaccagaa gtatagagtc cctgccagga ttggagctgg
1201 gcagtaggga gggaagagat ttatttcagg tgcctcagaa gataacttgc acctctgtag
1261 gatcacagtg gaagggtcat gctgggaagg agaagctgga gtcaccagaa aacccaatgg
1321 atgttgtgat gagccttact atttgtgtgg tcaatggggc ctactacttt ctctcaatcc
1381 tcacaactcc tggctcttaa taaccccaa aactttctct tctgcaggtc aagagaaagg
1441 atttctga [SEQ ID NO:30]
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```
1 aggacctgaa aaacgtgttc ccaccgagg tcgctgtgtt tgagccatca gaagcagaga
61 tctccacac ccaaaaggcc acactggtat gcctggccac aggtctctac cccgaccacg
121 tggagctgag ctggtgggtg aatgggaagg aggtgcacag tggggtcagc acagaccgcg
181 agccctcaa ggagcagccc gccctcaatg actccagata ctgcctgagc agcgcctga
241 gggctctggc caccttctgg cagaaccccc gcaaccactt ccgctgtcaa gtccagttct
```

301 acgggctctc ggagaatgac gagtggaccc aggatagggc caaacccgtc acccagatcg
 361 tcagcgccga ggcctggggg agagcagggt agtggggcct ggggagatgc ctggaggaga
 421 ttaggtgaga ccagctacca gggaaaatgg aaagatccag gtagcggaca agactagatc
 481 cagaagaaag ccagagtga caaggtggga tgatcaaggt tcacagggtc agcaaagcac
 5 541 ggtgtgact tccccacca agaagcatag aggtgaatg gagcacctca agctcattct
 601 tccttcagat cctgacacct tagagctaag ctttcaagtc tcctgagga ccagccatac
 661 agctcagcat ctgagtgggt tgcattccat tctcttcttg ggtcctggtt tcctaagatc
 721 atagtgacca cttcgtggc actggagcag catgaggag acagaaccag ggctatcaaa
 781 ggaggctgac tttgtactat ctgatatgca tgtgtttgtg gcctgtgagt ctgtgatgta
 10 841 aggtcaatg tccttcaaaa cgagcattct ctcatccatt tttcttcccc tgttttcttt
 901 cagactgtgg cttcacctcc ggtaagttag tctctccttt ttctctctat ctttcgcgt
 961 ctctgctctc gaaccagggc atggagaatc cacggacaca ggggctgag ggaggccaga
 1021 gccacctgtg cacaggtgcc tacatgctct gttcttgtca acagagtctt accagcaagg
 1081 ggtcctgtct gccaccatcc tctatgagat cttgctaggg aaggccacct tgtatgccgt
 1141 gctggtcagt gccctcgtgc tgatggccat ggtaaggagg aggggtggat agggcagatg
 1201 atgggggcag gggatggaac atcacacatg ggcataaagg aatctcagag ccagagcaca
 1261 gcctaataata tcctatcacc tcaatgaaac cataatgaag ccagactggg gagaaaatgc
 1321 agggaaatgc acagaatgca tcatgggagg atggagacaa ccagcgagcc ctactcaaat
 1381 taggcctcag agccgcctc ccctgcccta ctctgctgt gccatagccc ctgaaacct
 20 1441 gaaaatgttc tctctccac aggtcaagag aaaggattcc agaggctag
 [SEQ ID NO:31]

In accordance with the presently disclosed subject matter, an “TRBC nucleic acid molecule” refers to a polynucleotide encoding an TRBC polypeptide.

25 In certain embodiments, the constant domain of a presently disclosed HI-TCR
 comprises a native or modified TRGC peptide. In certain embodiments, the constant
 domain of a presently disclosed HI-TCR comprises a native or modified TRGC1 peptide.
 In certain embodiments, the TRGC1 polypeptide has an amino acid sequence that is at
 least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%
 30 or 100% homologous or identical to the amino acid sequence set forth in SEQ ID NO: 42,
 which is provided below.

DKQLDADVSP KPTIFLPSIA ETKLQKAGTY LCLLEKFFPD VIKIHWQEEK
 SNTILGSQEG NTMKTNDTYM KFSWLTVPKE SLDKEHRCIV RHENNKNGVD
 QEIIFFPIKT DVITMDPKDN CSKDANDTLL LQLTNTSAYY MYLLLLLKS
 35 VYFAIITCCL LRRTAFCCNG EKS [SEQ ID NO:42]

In certain embodiments, the constant domain of a presently disclosed HI-TCR
 comprises a native or modified TRGC2 peptide. In certain embodiments, the TRGC2
 polypeptide has an amino acid sequence that is at least about 85%, about 90%, about
 95%, about 96%, about 97%, about 98%, about 99% or 100% homologous or identical to
 40 the amino acid sequence set forth in SEQ ID NO: 43, which is provided below.

DKQLDADVSP KPTIFLPSIA ETKLQKAGTY LCLLEKFFPD IIKIHWQEEK
 SNTILGSQEG NTMKTNDTYM KFSWLTVPKE SLDKEHRCIV RHENNKNGID
 QEIIFFPIKT DVTTVDPKYN YSKDANDVIT MDPKDNWSKD ANDTLLQLT
 45 NTSAYYTYLL LLLKSVVYFA IITCCLLRRT AFCCNGEKS [SEQ ID NO:43]

In certain embodiments, the TRGC polypeptide has an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% homologous or identical to the amino acid sequence encoded by a transcript expressed by a gene of NCBI Genbank ID: 6966, NG_001336.2, range 108270 to 113860 (TRGC1, SEQ ID NO: 32), NCBI Genbank ID: 6967, NG_001336.2, range 124376 to 133924 (TRGC2, SEQ ID NO: 33) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

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1 ataaacaact tgatgcagat gtttccccca agcccactat ttttcttct tcaattgctg
61 aaacaaagct ccagaaggct ggaacatacc tttgtcttct tgagaaattt ttccctgatg
10 121 ttattaagat acattggcaa gaaaagaaga gcaacacgat tctgggatcc caggagggga
181 acaccatgaa gactaacgac acatacatga aatttagctg gttaacggtg ccagaaaagt
241 cactggacaa agaacacaga tgtatcgta gacatgagaa taataaaaaa ggagttgatc
301 aagaaattat ctttctcca ataaagacag gtatgtgttt acgcatatca tctgtcagaa
361 cacttctttg aaagtgaatg ctgcattttt tcctttcagt attaatgaaa aacaaacata
15 421 aatcttttct aaatattgtt acatttaatg gtagcataaa tgccctgcta cttttctata
481 gaattaaaaa ggtatagggt ttggagaaaa caaaattgaa aaagttagtg aaggtttgtc
541 agcctcagct ccattatcca aaataagaaa gtcacgtgct ggtttttagg gttgttagat
601 ggattaaaga aacaacatac acagaagcat ctagcaacgt gacacgtggt aaacgctcaa
661 aaagtgttct cccttctttt gatgacttta cttgatcagg aaataacata tatatgtctt
20 721 tcaggaatgt tctgcccagg caggagagtc actcacctca atcttgctac ccacaaagtt
781 taacctaaaa acaacgggtt cattgttgac aaaatgatgt ttatctgttg ttgacagaat
841 gatgtttatc taaaaacagt tccaattttt tatttctttt gctgagacac aaaggggagg
901 caaatgtgca aagcttgagg gtagtcttac cactgtgctt aagtgttctg atttttctag
961 tgatcagggc aaaataaaaa gtatagtaag ttccaaggca gtgaatatta tacaggagag
25 1021 aagttacagt tttataatgt gttttccttt acactaaatt ctaaaagtaa aaagtctttt
1081 tttttttttg acagagtttc actcttggtg cccaagcagg tgtgctatgg tatgatctca
1141 gctcactgca acctccacct cccgggttca agtgattctc ttacttcagc ctcccagacg
1201 gctgggattg caggcgcctg ccaccacacc tggctaattt ttgtgttttt agtagagatg
1261 ggggtttcac atgttgccca ggctggtctc aaattcctga cctcaagtga tccatccacc
30 1321 tcggcctcca agtgctggga ttatggcgct cagccactgt gccagccta aaagtaaaat
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1561 tctggtccac acttgcaacc aaccttgatt gagagagaga gagaactcag gataacttg
35 1621 aagattttat tatgggaac agttacttta ttctttttac ctcaatcaat gcatggaaat
1681 aagtgatagt cattttcatt tatcttttaa taaatgaagt caccatgagg aaaataaaaa
1741 gacattgaaa acccattaaa gtcagccctt aaagatattt ggacatgcag acttgataac
1801 taacgtttgc attcttgaga cttacccaaa acccatacct caagtccaag tttttagaat
1861 tcatgaaata aagatctcag tgagtgcata aaattgcgca ccagaatcat atccgtatag
40 1921 acaagaacac atctactaga aaaataataa accaacacac caatgcaact gtgttttctt
1981 ctgttttaaa gtatgttgct tttgtatgca tgtttgcttc ttcttttttt tttttaacat
2041 cacagataaa ttcaactctc acctcaggtt ttattgagag aactgtcaat gtgacttggc
2101 ctctgtcttt ctagtcccag aaagaattgc actgaaatct gagctcctgt aataaaaaa
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45 2221 cattatattg cctctttata aataactttc tatctatttc tgtggattat tcctacaaag
2281 tacttttcat atgtccaatt tcttttcttc ccctacaact actgtctgaa tactggctct
2341 gctatttgct gatatgattc tcggcaagtt gcctgcactt tttaaacttt atttcctcat
2401 tcagaacatg gggccataca taatacaact cacttcagtg ttattgggga attaaacaaa
2461 aaatgcatgg gaagcattta acatagtgcc tgacacaata atgagtactc agtagatgtt
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2581 gtacttgctg gggacattgg ggatatgcac gatttggaag agaagtactg ctttttttgc
2641 ttagatgaga aatttttcta agccagactc cttcaaatat gtaagattct gttgtggatt
2701 caaggactga aagaattctt ggccgagtgt ggtggcttat ccctgtaatc ccagcatttt
2761 gtgaggacaa ggcaggaaga ttgcttgagt ccaggagttt gaaaccagcc tgcgcaacat
55 2821 ggcgaaaccc tgtctctaca aaaaatacaa acattagctc ggagttagtg ctgacatgtg

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2881 cctgtactcc cagctactca gaaggctgag atgggaggat ctcatgagcc tggggagttt
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 3001 agaccctgcc tcaaaaaaca aacaaacaaa caaacaaaac aaaattaatc tttttgctga
 5 3061 tgtcatgtca gcagtggtg ttgaaggctg taaagcagcc atttgttcag tttatttttc
 3121 cattgaacaa gtatttatca aaaacatact ttgtggcagt cactatgcta ggagctatga
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 10 3421 cacacgaaca attgactagt acttgctctc attcttctag atgtcatcac aatggatccc
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 3601 tcattttggga ggacaaggca ggaagattgc ttgagcccag gacttggaag caagcctgga
 3661 caacatggcg aaaccctgtc tctacaaaaa atacaaacat tagctgggtca tgggagttag
 3721 tgctgtact cccagctact caggaggcta agataggagg atcacctgag cctgggcagt
 50 3781 ttgaggtttc agtgagccgt gatgacacca tactatactc cactccagcc tgggtgacag
 3841 tgacatcctg cctcaaaaaa acccccaaaa ttattctttt tgctgatttc atgtcagcag
 3901 tgtgtgctga aggtgtgaaa gtagccactt gttctgttta tttttccatt gaacaagtat
 3961 ttatcaaaaa cgtactttgt ggaaggcact gtgctaggaa ctatgcatac agaaggaaaa
 4021 ccaaatgttc ttggatacta cactccagtt gtgataaaaa agaaaaaagt attcttcaca
 55 4081 aacttcaaca ttttgatgtg caaaaacata atatatgaat tagatctacc taactacaca
 4141 gaattagacc aattatttct gggattatgg gctcatattt ttaataactg tctcctacc
 4201 tctctgttga caggttttct aaatattcat ttaattacac acagtcacag acacactcag
 4261 acacacacac atacacacac acacacacct tgacaaataa tgggcatgaa caattgactg
 4321 gtacttctct tcattcttct acagtgcacc acagtggatc ccaaatacaa ttattcaag
 60 4381 gatgcaaatg gtaagttttt gtgtttttta tttcctcctg atcattttaa gttttgaact
 4441 tctctggctt gaaaaatcag ggaatggatt ttgctaggtt ggatgctgca gaatggacct

4501 aatcatatatt taaatttagtc cctcttttttc taggagttgt attaacaac ctaactactg
 4561 cttcatgtaa gagatgactg taaattgaag ggtacagtga tatgctttca gttattttcaa
 4621 aaaacagact ttactcatcc atgtgtctttt tttcttttct tttttttctt ttttgagacg
 5 4681 gagtctcgct ctgttgaaca ggctggattg cagtgcgcg atctcacctc actacaacct
 4741 ccgcctctgg agttcaagcg attctccagc ctcagcttct caagtagctg ggactacagg
 4801 cacatgccac catgtccggg tcatctttgt attttttagca gagaccgggt ttcactatgt
 4861 tggccaggct ggtctagaat tcctgacttc gtgatctgcc ccctcagccc tccgaagtgc
 4921 tgggattaca gacgtgagtc actgtgcccg gcctaacagt aaaatgtctt tcatgcgctt
 4981 ctcaaggcaa ctacgttaag gaggacactt ctcttaatgt cattctacag tagattttcta
 10 5041 atgctctttc ttggaagttt gtttttctga gaaaagctaa aaatataaca tgggaagtgt
 5101 catattgtat aatcaatgaa gtgcttttca aggagataaa actaatctgg tccacgtttg
 5161 caaccaacct tgattgagag agagagagaa ctcaggatac acttgagat tttattatgg
 5221 ggaatagtta ctttattctt ttttctcaa tcaattcatg gaaataagt atagtcatat
 5281 tcatttatct tttataaat gaagtcacca tgaggaaaat aaaaagacat tgaaccacca
 15 5341 ttaagtttag cccttaaaga tatttgga caactgtgtt ttcttctggt ttaaaatag
 5401 tgagacttac ccaaacacca tacctcaagt ccatgttttt agaattcatg aaataaagat
 5461 ctcaagttagt gcataaaatt gcgcaccaga atcataatccg tatagacaag aacacatcta
 5521 ctagaaaaat aataaaccaa cacaccaatg caactgtgtt ttcttctggt ttaaaatag
 5581 ttgtctttgt atgcatgttt gcttcttctt tttttttttt taacatcaca gataaattca
 20 5641 actctcacct caggttttat tgagagaact gtcaatgtga cttggcctct gtctttctag
 5701 tcccagaaag aatcgactg aaatgctgag ctctgtaat aaaaatgacc atttgctgag
 5761 agtaattaac atactgaaag agattttctt agagtacaca atggtgacat tataattgtct
 5821 ctttataaat aactttctat ctatttctgt ggattattcc taaaaagta ttttcatatg
 5881 tccagtttct tttcttcccc tacaactacc gtctgaatag tggctctgct atttgctgat
 25 5941 atgattctcg gcaagttgcc tgcacttttt aaactttatt tcctcattca gaacatgggg
 6001 ccatgtaata ctcatgtacg tgagtattac gtaataatgc tcacttaagt gttactgggg
 6061 aattaaacaa aaaaatgcat ggcaagcatt taacatagtg cctgacacaa taatgagcac
 6121 tcagtagatg ttagatttta ttaatatgtt tgttgttatg tccggaacaa ctataacctc
 6181 agaaatcat gggtacttgc ttgggatgtt ggggatatgc atgatttggg aaggtatgac
 30 6241 tgcttttttc tgcttagatg agaaattttt ctaagccaga ctccctcaaa tatgtaagat
 6301 tctgttgtgg attctaggac ggaaagaatt cttgggtcagg tgtggtttct tatccctgta
 6361 atcccagaat tttgggagga caaggcagga agattgcttg agcccaggag tttgaaacca
 6421 gcctgggcaa caagacgaaa ccctgtctct acaaaagtac ataaattagc ttggcttggt
 6481 ggtgtgtgcc tgtattacca gctattcggg agactgagat gggaggatct cctgaacctg
 35 6541 tgaagtttga ggcttcagt agccgtgatg acaccatact atactcgact ccagctctag
 6601 cgacagttag actctgcgtc aaaaaaaaatt ccccaaaatt attgtttttg ctgatttctg
 6661 gtcagcagtg tgtgctgaag ggtgtaaagt agccacttga tcagtttatt tttccactga
 6721 acaagtattt atcaaaaaca tactttgtgg tctgtttttg ataaataaaa aggcactgtg
 6781 ctaggagcca tgaatacaga aggaaaacca aatgttcttg gatactacac tccagttgtg
 40 6841 ataaaaaaga aaaaatgtatt cttcacgaac ttcaacattt tgatatgcaa aaacatagta
 6901 ctataaattag atctacctga ttacgtagaa tcagaccaat tatttctgga attgaggct
 6961 catattttta ataactgtcc tcctgcctct ctggtgacag gttttataaa tattcattta
 7021 attacacaca cacacacaca caccttgaca aataatggac atgaacaatt gactagtact
 7081 tgctctcatt cttctagatg tcatcacaaat ggatcccaaa gacaattggg caaaagatgc
 45 7141 aaatggtaag cttttgtgtt tttcctttcc tcctgatcat ttttaagttt gaacttctct
 7201 ggcttgaaaa atcagggaat ggccgggtg cgggtggctca cgcctgtaat ccagcactt
 7261 tgggagggcg agcgggcggt atcacgaggt caggagatcg agaccatccc ggctaaaacg
 7321 gtgaaacccc gtctctacta aaaatacaaa aaattagccg ggcttagtgg cggcgccctg
 7381 tagtcccagc tacttgggag gctgaggcag gagaatggcg tgaacccggg aggcggagct
 50 7441 tgcaagttag cgagattgag ccactgcact ccactccagc ctgggcgaca gagcgagact
 7501 ccgtctcaaa aaaaaaaaaa aaaaaaaaaa aagaaaaatc agggaatgga ttttgctagg
 7561 ttgatgctg cagaatggac ctagtgtat ttttaattag tccctctttt tctaggagtt
 7621 gtattaacaa acctaactac tgcttcgggt atgagatgac tgtaaatag aggttacagt
 7681 gatagtcttt cagttatttc aaaaaacaga ctttattcat ccgtctgtct tttttttttt
 55 7741 tttttttttt tttttttgag acggaggagt ctcactctat caccagggt ggagtgcagt
 7801 ggcgcatct cggctcacca taacctccgc cttactggtt caagcgattc tccagcctca
 7861 gcttctcaag tagctgggac tacaggtgca caccaccata cctggcta at ttttgtaatt
 7921 ttaatagaga tggggtttca caacgtggc caggatggct ttgaattctt gacctcgtga
 7981 tctgccccct cgggtctcca aactctggg attataggcg tgagccactg tgcccgct
 60 8041 tctgtctttt gttataatga ctggggaaaa catgatacca tgttgcttct tgagtgtttt
 8101 tgttttagtc tttgtctttt gctagtagct aataacacga actagtgttt atcaagtgtc

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8161 ttttacacag aagggccttgt tctgcatttt ctagtttaat catcttaata ctccataaaa
8221 gtagtacaat atattttctc ccattttaca gtccctttaa agtaaataac tataaaaaatc
8281 ccttatacat gtcacacagc taggtctggc atttcaaatac aggacatcaa acaaagaatt
8341 cgtgcagtta ctaagtcctc tattttttct acaatagaaa aaatagcaag aattacagat
5 8401 agcaagacat tacaaggcag gaatctgaaa cgaaaggac ataatgtggg gctgggtggg
8461 tgcattgagct ttgcagacta gactttcatt ccagctcttt taatgattag gtgtaagtga
8521 cctacatttt gtgagtaaca gttttctcat cagccaacta agaataatta caccagattc
8581 acagtatttg aagagataag ggcattgaatg tgagatgtct ggcgtagggt atctcattta
8641 gcagacacag aatgaatact tgtttctggc tttttctctc tacatatgca caaagaatgt
10 8701 gactagaagc attggctcta gccctgctca actttcctct atttccaata ccaaggggct
8761 ctgacttagg ctgccacacc aggcaaggag gggcagtacc acctcacttg accaagggca
8821 gggagtcacg gacacatcac ttcttgagat ccttttccac accaaggact gatgtttctg
8881 gaattctcac tttatgaaga caaacatat aaatggaaat ttctgcagga agagactcac
8941 tctttagtag cattgagtag gcactagtgg tccaccccca ctgtctttac ttattccttg
15 9001 acatcacata tctcttgtaa aacctcaaat aatgttaaat gcaatcacc aataatagca
9061 tagccataat tagaggcatt taggaaagac aggtgagtgt gccacaacta cctaacacat
9121 cagcaaactc ggattaacca ctttctttga ttttccaca tgcaacctta ctttttaata
9181 gttggggaatg ttctaagtga atttagcaga ggttgtaaat caacttgaaa gctgaattct
9241 gacttgtctg actcttggtg gtgctggtag cagtagatgt ttacttttag gttttggtgg
20 9301 tggtggaata tcacttcaac gtaaatcatc agaaataagt atttggaac ccctctcgca
9361 ttaatatatc ttattctgta aaaagaacat gtgcaatttc tcttagatac actactgctg
9421 cagctcacia acacctctgc atattacacg tacctcctcc tgctcctcaa gagtgggtgc
9481 tatttttgcca tcatcacctg ctgtctgctt agaagaacgg ctttctgctg caatggagag
9541 aaatcataa [SEQ ID NO:33]
25

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In accordance with the presently disclosed subject matter, an “TRGC nucleic acid molecule” refers to a polynucleotide encoding an TRGC polypeptide.

In certain embodiments, the constant domain of a presently disclosed HI-TCR comprises a native or modified TRDC peptide. In certain embodiments, the TRDC polypeptide has an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% homologous or identical to the amino acid sequence set forth in SEQ ID NO: 41, which is provided below.

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35 SQPHTKPSVF VMKNGTNVAC LVKEFYPKDI RINLVSSKKI TEFDP AIVIS
PSGKYNAVKL GKYEDSNSVT CSVQHDNKT V HSTD F EVKTD STDHV KPKET
ENTKQPSKSC HKPKAIVHTE KVNMSLTVL GLRMLFAKT V AVNFL LTAKL
FFL [SEQ ID NO:41]

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In certain non-limiting embodiments, T cell receptor constant region comprises a hinge/spacer region that links the extracellular antigen-binding domain to the constant domain. The hinge/spacer region can be flexible enough to allow the antigen binding domain to orient in different directions to facilitate antigen recognition. In certain non-limiting embodiments, the hinge/spacer region can be the hinge region from IgG1, or the CH₂CH₃ region of immunoglobulin and portions of CD3, a portion of a CD28 polypeptide, a portion of a CD8 polypeptide, a variation of any of the foregoing which is at least about 80%, at least about 85%, at least about 90%, or at least about 95% homologous or identical thereto, or a synthetic spacer sequence. In certain non-limiting

embodiments, the hinge/spacer region of the CAR can comprise a native or modified hinge region of a CD3 ζ polypeptide, a CD40 polypeptide, a 4-1BB polypeptide, an OX40 polypeptide, a CD166 peptide, a CD166 peptide, a CD8a peptide, a CD8b peptide, an ICOS polypeptide, an ICAM-1 peptide, a CTLA-4 peptide, a synthetic peptide (not based
 5 on a protein associated with the immune response), or a combination thereof.

2.4. Intracellular Signaling Domain

In certain non-limiting embodiments, a presently disclosed HI-TCR comprises an antigen binding chain, which does not comprise an intracellular domain. In certain embodiments, the antigen binding chain is capable of associating with a CD3 ζ
 10 polypeptide. In certain embodiments, the antigen binding chain comprises a constant domain, which is capable of associating with a CD3 ζ polypeptide. In certain embodiments, the CD3 ζ polypeptide is endogenous. In certain embodiments, the CD3 ζ polypeptide is exogenous. In certain embodiments, binding of the antigen binding chain to an antigen is capable of activating the CD3 ζ polypeptide associated to the antigen
 15 binding chain. In certain embodiments, the exogenous CD3 ζ polypeptide is fused to or integrated with a costimulatory molecule disclosed herein.

In certain non-limiting embodiments, a presently disclosed HI-TCR comprises an antigen binding chain that comprises an intracellular domain. In certain embodiments, the intracellular domain comprises a CD3 ζ polypeptide. In certain embodiments, binding
 20 of the antigen binding chain to an antigen is capable of activating the CD3 ζ polypeptide of the antigen binding chain.

The activated CD3 ζ polypeptide can activate and/or stimulate an immunoresponsive cell (*e.g.*, a cell of the lymphoid lineage, *e.g.*, a T cell). CD3 ζ comprises three immunoreceptor tyrosine-based activation motifs (ITAM1, ITAM2 and
 25 ITAM3), three basic-rich stretch (BRS) regions (BRS1, BRS2 and BRS3), and transmits an activation signal to the cell (*e.g.*, a cell of the lymphoid lineage, *e.g.*, a T cell) after antigen is bound to the antigen binding chain. The intracellular signaling domain of the CD3 ζ -chain is the primary transmitter of signals from endogenous TCRs. In certain embodiments, the CD3 ζ polypeptide comprises or has an amino acid sequence that is at
 30 least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous to the sequence having a NCBI Reference No: NP_932170 (SEQ ID NO: 17), NCBI Reference No: NP_000725.1 (SEQ ID NO: 18) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In certain non-limiting embodiments, the CD3 ζ polypeptide

comprises or has an amino acid sequence that is a consecutive portion of SEQ ID NO: 17, which is at least 20, or at least 30, or at least 40, or at least 50, and up to 164 amino acids in length. Alternatively or additionally, in non-limiting various embodiments, the CD3 ζ polypeptide comprises or has an amino acid sequence of amino acids 1 to 164, 1 to 50, 50 to 100, 100 to 150, or 150 to 164 of SEQ ID NO: 17. In certain embodiments, the CD3 ζ polypeptide comprises or has an amino acid sequence of amino acids 52 to 164 of SEQ ID NO: 17.

SEQ ID NO: 17 is provided below:

1 MKWKALFTAA ILQAQLPITE AQSFGLLDPK LCYLLDGILF IYGVILTALF LRVKFSRSAD
10 61 APAYQQGQNQ LYNELNLGR EEYDVLDRR GRDPEMGGKP QRRKNPQEG LYNELQDKMA
121 EAYSEIGMKG ERRRGKGDG LYQGLSTATK DTYDALHMQA LPPR [SEQ ID NO: 17]

In certain embodiments, the intracellular signaling domain comprises a human CD3 ζ polypeptide. The human CD3 ζ polypeptide can comprise or have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous or identical to SEQ ID NO: 18 or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. SEQ ID NO: 18 is provided below:

RVKFSRSADA PAYQQGQNQL LYNELNLGR EEYDVLDRR GRDPEMGGKP RKNPQEGLYN
15 ELQDKMAEA YSEIGMKG RRGKGDGLY QGLSTATKDT YDALHMQALP PR [SEQ ID NO:
20 18].

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 18 is set forth in SEQ ID NO: 19, which is provided below.

AGAGTGAAGTT CAGCAGGAGCGCAGACGCCCCGCGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTC
AATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGAAAG
25 CCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGT
GAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGCTCTCAGTACAGCC
ACCAAGGACACCTACGACGCCCTTACATGCAGGCCCTGCCCCCTCGC [SEQ ID NO: 19]

2.4.1. Co-stimulatory region

In certain non-limiting embodiments, a presently disclosed HI-TCR comprises an antigen binding chain that comprises an intracellular domain, wherein the intracellular domain comprises a co-stimulatory region. In certain embodiments, the intracellular domain comprises a co-stimulatory region and a CD3 ζ polypeptide. In certain embodiments, the intracellular domain comprises a co-stimulatory region and does not comprise a CD3 ζ polypeptide.

In certain embodiments, the co-stimulatory region comprises at least one co-stimulatory molecule, which can provide optimal lymphocyte activation. As used herein, “co-stimulatory molecules” refer to cell surface molecules other than antigen receptors or their ligands that are required for an efficient response of lymphocytes to antigen. The at least one co-stimulatory signaling region can include a CD28 polypeptide, a 4-1BB polypeptide, an OX40 polypeptide, an ICOS polypeptide, a DAP-10 polypeptide, or a combination thereof. The co-stimulatory molecule can bind to a co-stimulatory ligand, which is a protein expressed on cell surface that upon binding to its receptor produces a co-stimulatory response, *i.e.*, an intracellular response that effects the stimulation provided when an antigen binds to its CAR molecule. Co-stimulatory ligands, include, but are not limited to CD80, CD86, CD70, OX40L, and 4-1BBL. As one example, a 4-1BB ligand (*i.e.*, 4-1BBL) may bind to 4-1BB (also known as “CD137”) for providing an intracellular signal that in combination with a CAR signal induces an effector cell function of the CAR⁺ T cell. CARs comprising an intracellular signaling domain that comprises a co-stimulatory signaling region comprising 4-1BB, ICOS or DAP-10 are disclosed in U.S. 7,446,190, which is herein incorporated by reference in its entirety.

In certain embodiments, the co-stimulatory region of an antigen binding chain of a HI-TCR comprises a co-stimulatory signaling region that comprises a CD28 polypeptide. The CD28 polypeptide can comprise or have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% homologous or identical to the sequence having a NCBI Reference No: P10747 or NP_006130 (SEQ ID NO: 20), or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. In non-limiting certain embodiments, the CD28 polypeptide comprises or has an amino acid sequence that is a consecutive portion of SEQ ID NO: 20 which is at least 20, or at least 30, or at least 40, or at least 50, and up to 220 amino acids in length. Alternatively or additionally, in non-limiting various embodiments, the CD28 polypeptide comprises or has an amino acid sequence of amino acids 1 to 220, 1 to 50, 50 to 100, 100 to 150, 114 to 220, 150 to 200, or 200 to 220 of SEQ ID NO: 20. In certain embodiments, the co-stimulatory region comprises a co-stimulatory signaling region that comprises a CD28 polypeptide comprising or having an amino acid sequence of amino acids 180 to 220 of SEQ ID NO: 20.

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1  MLRLLLALNL FPSIQVTGNK ILVKQSEPLV AYDNAVNLSC KYSYNLFSRE FRASLHKGLD
61  SAVEVCVVYG NYSQQLQVYS KTGFNCDGKL GNEVTFYLLQ NLYVNQTDIY FCKIEVMYPP

```

121 PYLDNEKSNG TIIHVKGKHL CPSPLFPGPS KPFWVLVVVG GVLACYSLLV TVAFIIFWVR
181 SKRSRLHSD YNMNTPRRPG PTRKHYQPYA PPRDFAAYRS [SEQ ID NO: 20]

In certain embodiments, the co-stimulatory region comprises a human intracellular signaling domain of CD28. The human intracellular signaling domain of CD28 can
5 comprise or have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous or identical to SEQ ID NO: 21 or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. SEQ ID NO: 21 is provided below:

10 RSKRSRLHSD YNMNTPRRPG PTRKHYQPYA PPRDFAAYRS [SEQ ID NO: 21].

An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 21 is set forth in SEQ ID NO: 22, which is provided below.

AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGCCGCCCGGGGCCACCCGC
AAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAGCCTATCGCTCC [SEQ ID NO: 22]

15 In certain embodiments, the co-stimulatory region comprises a co-stimulatory signaling region that comprises two co-stimulatory molecules, e.g., co-stimulatory signaling regions of CD28 and 4-1BB or co-stimulatory signaling regions of CD28 and OX40.

4-1BB can act as a tumor necrosis factor (TNF) ligand and have stimulatory
20 activity. The 4-1BB polypeptide can comprise or have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous or identical to the sequence having a NCBI Reference No: P41273 or NP_001552 (SEQ ID NO: 23) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

25 SEQ ID NO: 23 is provided below:

1 MGNSCYNIVA TLLLVLNFER TRSLQDPCSN CPAGTFCDNN RNQICSPCPP NSFSSAGGQR
61 TCDICRQCKG VFRTRKECSS TSNAECDCTP GFHCLGAGCS MCEQDCKQGQ ELTKKGCKDC
121 CFGTFNDQKR GICRPWTNCS LDGKSVLVNG TKERDVVCGP SPADLSPGAS SVTPPAPARE
181 PGHSPQIISF FLALTSTALL FLLFFLTLEF SVVKRGRKKL LYIFKQPFMR PVQTTQEEDG
30 241 CSCRFPPEEEE GGCEL [SEQ ID NO: 23]

In accordance with the presently disclosed subject matter, a “4-1BB nucleic acid molecule” refers to a polynucleotide encoding a 4-1BB polypeptide.

In certain embodiments, the co-stimulatory region comprises an intracellular signaling domain of 4-1BB. The intracellular signaling domain of 4-1BB can comprise or
35 have an amino acid sequence that is at least about 85%, about 90%, about 95%, about

96%, about 97%, about 98%, about 99% or about 100% homologous or identical to SEQ ID NO: 24 or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions. SEQ ID NO: 24 is provided below:

KRGRKKLLYI FKQPFMRPVQ TTQEEDGCSC RFPEEEEGGC EL [SEQ ID NO: 24].

- 5 An exemplary nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 24 is set forth in SEQ ID NO: 27, which is provided below.

AAACGGGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACTCA
AGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAGGATGTGAACTG [SEQ ID
NO: 27]

- 10 An OX40 polypeptide can comprise or have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous or identical to the sequence having a NCBI Reference No: P43489 or NP_003318 (SEQ ID NO: 25), or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

- 15 SEQ ID NO: 25 is provided below:

1 MCVGARRLGR GPCAAALLLG LGLSTVTGLH CVGDTYPSND RCHECRPGN GMVSRCSRSQ
61 NTVCRPCGPG FYNDVVSSKP CKPCTWCNLR SGSEKQLCT ATQDTVCRRCR AGTQPLDSYK
121 PGVDCAPCFP GHFSPGDNQA CKPWTNCTLA GKHTLQPASN SSDAICEDRD PPATQPQETQ
181 GPPARPITVQ PTEAWPRTSQ GPSTRPVEVP GGRAVAAILG LGLVLGLLGP LAILLALYLL
20 241 RRDQRLPPDA HKPPGGGSFR TPIQEEQADA HSTLAKI [SEQ ID NO: 25]

In accordance with the presently disclosed subject matter, an “OX40 nucleic acid molecule” refers to a polynucleotide encoding an OX40 polypeptide.

- An ICOS polypeptide can comprise or have an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or
25 about 100% homologous or identical to the sequence having a NCBI Reference No: NP_036224 (SEQ ID NO: 26) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

SEQ ID NO: 26 is provided below:

1 MKSGLWYFFL FCLRIKVLTG EINGSANYEM FIFHNGGVQI LCKYPDIVQQ FKMQLLKGGQ
30 61 ILCDLTKTKG SGNTVSIKSL KFCHSQLSNN SVSFFLYNLD HSHANYFCN LSIFDPPPFK
121 VTLTGGYLHI YESQLCCQLK FWLPIGCAAF VVVCILGCIL ICWLTKKKYS SSVHDPNGEY
181 MFMRAVNTAK KSRLTDVTL [SEQ ID NO: 26]

In accordance with the presently disclosed subject matter, an “ICOS nucleic acid molecule” refers to a polynucleotide encoding an ICOS polypeptide.

- 35 In certain embodiments, mutation sites and/or junction between domains/motifs/regions of the CAR derived from different proteins are de-immunized.

Immunogenicity of junctions between different CAR moieties can be predicted using NetMHC 4.0 Server. For each peptide containing at least 1 aa from next moiety, binding affinity to HLA A, B and C, for all alleles, can be predicted. A score of immunogenicity of each peptide can be assigned for each peptide. Immunogenicity score can be calculated using the formula Immunogenicity score=[(50-binding affinity)*HLA frequency]ⁿ. n is the number of prediction for each peptide.

2.5. CD3 Complex

In certain embodiments, a presently disclosed HI-TCR is capable of associating with a CD3 complex (also known as “T-cell co-receptor”). In certain embodiments, the HI-TCR and the CD3 complex form an antigen recognizing receptor complex similar to a native TCR/CD3 complex. In certain embodiments, the CD3 complex is endogenous. In certain embodiments, the CD3 complex is exogenous. In certain embodiments, the presently disclosed HI-TCR replaces a native and/or an endogenous TCR in the CD3/TCR complex. In certain embodiments, the CD3 complex comprises a CD3γ chain, a CD3δ chain, and two CD3ε chains.

In certain embodiments, the CD3γ chain comprises or has an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous or identical to NCBI reference number: NP_000064.1 (SEQ ID NO: 34, which is provided below) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

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1 meqgkglavl ilaiillqgt laqsikgnhl vkvydyqedg svlltcdaea knitwfkdgk
61 migfltedkk kwnlgsnakd prgmyqckgs qnkskplqvy yrmcqniciel naatisgflf
121 aeivsifvla vgvyfiagqd gvrqsrask qtllpndqly qplkdreddq yshlqgnqlr
181 rn [SEQ ID NO:34]
```

In certain embodiments, the CD3δ chain comprises or has an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% homologous or identical to NCBI reference numbers: NP_000723.1 (SEQ ID NO: 35, which is provided below), NP_001035741.1 (SEQ ID NO: 36, which is provided below) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

```

1 mehstflsgl vlatllsqvs pfkipieele drvfvnnts itwvegtvgt llsditrldl
61 gkrildprgi yrcngtdiyk dkestvqvhy rmcqscveld patvagiivt dviatlallal
121 gvfcfaghet grlsgaadtq allrndqvyy plrdrrdaqy shlggnwarn k [SEQ ID NO:
35]
```

```

1 mehstflsgl vlatllsqvs pfkipieele drvfvnnts itwvegtvgt llsditrldl
```

61 gkrildprgi yrcngtdiyk dkestvqvhy rtadtqallr ndqvyqplrd rddaqyshlg
121 gnwarnk [SEQ ID NO: 36]

In certain embodiments, the CD3ε chain comprises or has an amino acid sequence that is at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%,
5 about 99% or about 100% homologous or identical to NCBI reference number: NP_000724.1 (SEQ ID NO: 37, which is provided below) or fragments thereof, and/or may optionally comprise up to one or up to two or up to three conservative amino acid substitutions.

10 1 mqsqthwrvl glcllsvgvw gqdgneemgg itqtpykvs i sgttviltcp qypgseilwq
61 hndkniggde ddknigsded hlsikefsel eqsgyyvcyp rgskpedanf ylylrarvce
121 ncmemdvmsv ativivdici tgglllllvyw wsknrkakak pvtrgagagg rqrqgnkerp
181 ppvpnpdyep irkgqrdlys glnqrri [SEQ ID NO: 37]

In certain embodiments, the recombinant TCR exhibits a greater antigen
15 sensitivity than a CAR targeting the same antigen. In certain embodiments, the recombinant TCR is capable of inducing an immune response when binding to an antigen that has a low density on the surface of a tumor cell. In certain embodiments, immunoresponsive cells comprising a presently disclosed HI-TCR can be used to treat a
20 subject having tumor cells with a low expression level of a surface antigen, e.g., from a relapse of a disease, wherein the subject received treatment which leads to residual tumor cells. In certain embodiments, the tumor cells have a low density of a target molecule on the surface of the tumor cells. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 5,000 molecules per cell, less than about 4,000 molecules per cell, less than about 3,000 molecules per cell, less than
25 about 2,000 molecules per cell, less than about 1,500 molecules per cell, less than about 1,000 molecules per cell, less than about 500 molecules per cell, less than about 200 molecules per cell, or less than about 100 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 2,000 molecules per cell. In certain embodiments, a target molecule having a low density
30 on the cell surface has a density of less than about 1,500 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 1,000 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of between about 4,000 molecules per cell and about 2,000 molecules per cell, between about 2,000 molecules per cell and
35 about 1,000 molecules per cell, between about 1,500 molecules per cell and about 1,000 molecules per cell, between about 2,000 molecules per cell and about 500 molecules per

cell, between about 1,000 molecules per cell and about 200 molecules per cell, or between about 1,000 molecules per cell and about 100 molecules per cell.

2.6. HI-TCR 19

In certain embodiments, a presently disclosed HI-TCR comprises two antigen
5 binding chains, e.g., VL-TRAC and VH-TRBC, which are capable of dimerizing, wherein the HI-TCR binds to CD19 (e.g., human CD19).

VL-TRAC

In certain embodiments, a presently disclosed HI-TCR comprises an antigen
binding chain that comprises an extracellular antigen-binding domain of a V_L domain of
10 an antibody and a constant domain of TRAC. In certain embodiments, the antibody binds to CD19 (e.g., human CD19). In certain embodiments, the antigen binding chain is designated as “VL-TRAC”.

VH-TRBC

In certain embodiments, a presently disclosed HI-TCR comprises an antigen
15 binding chain that comprises an extracellular antigen-binding domain of a V_H domain of an antibody and a constant domain of TRBC. In certain embodiments, the antibody binds to CD19 (e.g., human CD19). In certain embodiments, the antigen binding chain is designated as “VH-TRBC”.

3. Immunoresponsive Cells

20 The presently disclosed subject matter provides immunoresponsive cells comprising a presently disclosed HI-TCR. In certain embodiments, the HI-TCR is capable of activating the immunoresponsive cell. Upon binding to the antigen, the immunoresponsive cells exhibit cytolytic effects towards cells bearing the antigen. In certain embodiments, the immunoresponsive cells comprising the HI-TCR exhibits
25 comparable or better therapeutic potency compared to cells comprising a chimeric antigen receptor (CAR) targeting the same antigen. In certain embodiments, the immunoresponsive cells comprising the HI-TCR exhibit comparable or better cytolytic effects compared to cells comprising a chimeric antigen receptor (CAR) targeting the same antigen. In certain embodiments, the immunoresponsive cells comprising the HI-
30 TCR secrete anti-tumor cytokines. The cytokines secreted by the immunoresponsive cells include, but are not limited to, TNF α , IFN γ and IL2.

The immunoresponsive cells of the presently disclosed subject matter can be cells of the lymphoid lineage. The lymphoid lineage, comprising B, T and natural killer (NK) cells, provides for the production of antibodies, regulation of the cellular immune system,

detection of foreign agents in the blood, detection of cells foreign to the host, and the like. Non-limiting examples of immunoresponsive cells of the lymphoid lineage include T cells, Natural Killer T (NKT) cells, and precursors thereof including embryonic stem cells, and pluripotent stem cells (*e.g.*, those from which lymphoid cells may be differentiated). T cells can be lymphocytes that mature in the thymus and are chiefly responsible for cell-mediated immunity. T cells are involved in the adaptive immune system. The T cells of the presently disclosed subject matter can be any type of T cells, including, but not limited to, helper T cells, cytotoxic T cells, memory T cells (including central memory T cells, stem-cell-like memory T cells (or stem-like memory T cells), and two types of effector memory T cells: *e.g.*, T_{EM} cells and T_{EMRA} cells, Regulatory T cells (also known as suppressor T cells), Natural killer T cells, Mucosal associated invariant T cells, and $\gamma\delta$ T cells. Cytotoxic T cells (CTL or killer T cells) are a subset of T lymphocytes capable of inducing the death of infected somatic or tumor cells. A patient's own T cells may be genetically modified to target specific antigens through the introduction of an HI-TCR. In certain embodiments, the immunoresponsive cell is a T cell. The T cell can be a CD4⁺ T cell or a CD8⁺ T cell. In certain embodiments, the T cell is a CD4⁺ T cell. In certain embodiments, the T cell is a CD8⁺ T cell.

In certain embodiments, the immunoresponsive cell comprises an exogenous or a recombinant (*e.g.*, the cell is transduced with) at least one co-stimulatory ligand. In certain embodiments, the immunoresponsive cell co-expresses the HI-TCR and the at least one exogenous co-stimulatory ligand. The interaction between the HI-TCR and at least one exogenous co-stimulatory ligand provides a non-antigen-specific signal important for full activation of an immunoresponsive cell (*e.g.*, T cell). Co-stimulatory ligands include, but are not limited to, members of the tumor necrosis factor (TNF) superfamily, and immunoglobulin (Ig) superfamily ligands. TNF is a cytokine involved in systemic inflammation and stimulates the acute phase reaction. Its primary role is in the regulation of immune cells. Members of TNF superfamily share a number of common features. The majority of TNF superfamily members are synthesized as type II transmembrane proteins (extracellular C-terminus) containing a short cytoplasmic segment and a relatively long extracellular region. TNF superfamily members include, but are not limited to, nerve growth factor (NGF), CD40L (CD40L)/CD154, CD137L/4-1BBL, TNF- α , CD134L/OX40L/CD252, CD27L/CD70, Fas ligand (FasL), CD30L/CD153, tumor necrosis factor beta (TNF β)/lymphotoxin-alpha (LT α),

lymphotoxin-beta (LT β), CD257/B cell-activating factor (BAFF)/Blys/THANK/Tall-1, glucocorticoid-induced TNF Receptor ligand (GITRL), and TNF-related apoptosis-inducing ligand (TRAIL), LIGHT (TNFSF14). The immunoglobulin (Ig) superfamily is a large group of cell surface and soluble proteins that are involved in the recognition, binding, or adhesion processes of cells. These proteins share structural features with immunoglobulins -- they possess an immunoglobulin domain (fold). Immunoglobulin superfamily ligands include, but are not limited to, CD80 and CD86, both ligands for CD28. In certain embodiments, the at least one co-stimulatory ligand is selected from the group consisting of 4-1BBL, CD275, CD80, CD86, CD70, OX40L, CD48, TNFRSF14, and combinations thereof. In certain embodiments, the immunoresponsive cell comprises or consists of one exogenous or recombinant co-stimulatory ligand. In certain embodiments, the one exogenous or recombinant co-stimulatory ligand is 4-1BBL or CD80. In certain embodiments, the one exogenous or recombinant co-stimulatory ligand is 4-1BBL. In certain embodiments, the immunoresponsive cell comprises or consists of two exogenous or recombinant co-stimulatory ligands. In certain embodiments, the two exogenous or recombinant co-stimulatory ligands are 4-1BBL and CD80.

In certain embodiments, the immunoresponsive cell can comprise or be transduced with at least one chimeric co-stimulatory receptor (CCR). As used herein, the term "chimeric co-stimulatory receptor" or "CCR" refers to a chimeric receptor that binds to an antigen, and, upon its binding to the antigen, provides a co-stimulatory signal to a cell (e.g., a T cell) comprising the CCR, but does not alone provide an activation signal to the cell. CCR is described in Krause, et al., J. Exp. Med. (1998);188(4):619-626, and US20020018783, which is incorporated by reference in its entirety. CCRs mimic co-stimulatory signals, but unlike, CARs, do not provide a T-cell activation signal, e.g., CCRs lack a CD3 ζ polypeptide. CCRs provide co-stimulation, e.g., a CD28-like signal, in the absence of the natural co-stimulatory ligand on the antigen-presenting cell. A combinatorial antigen recognition, i.e., use of a CCR in combination with a CAR, can augment T-cell reactivity against the dual-antigen expressing T cells, thereby improving selective tumor targeting. See WO2014/055668, which is incorporated by reference in its entirety. Kloss et al., describe a strategy that integrates combinatorial antigen recognition, split signaling, and, critically, balanced strength of T-cell activation and co-stimulation to generate T cells that eliminate target cells that express a combination of antigens while sparing cells that express each antigen individually (Kloss et al., Nature Biotechnology (2013);31(1):71-75, the content of which is incorporated by reference in

its entirety). With this approach, T-cell activation requires CAR-mediated recognition of one antigen, whereas co-stimulation is independently mediated by a CCR specific for a second antigen. To achieve tumor selectivity, the combinatorial antigen recognition approach diminishes the efficiency of T-cell activation to a level where it is ineffective without rescue provided by simultaneous CCR recognition of the second antigen.

In certain embodiments, the CCR comprises an extracellular antigen-binding domain that binds to a second antigen, a transmembrane domain, and a co-stimulatory signaling region that comprises at least one co-stimulatory molecule. In certain embodiments, the CCR does not alone deliver an activation signal to the cell. Non-limiting examples of co-stimulatory molecules include CD28, 4-1BB, OX40, ICOS, DAP-10 and any combination thereof. In certain embodiments, the co-stimulatory signaling region of the CCR comprises one co-stimulatory signaling molecule. In certain embodiments, the one co-stimulatory signaling molecule is CD28. In certain embodiments, the one co-stimulatory signaling molecule is 4-1BB. In certain embodiments, the co-stimulatory signaling region of the CCR comprises two co-stimulatory signaling molecules. In certain embodiments, the two co-stimulatory signaling molecules are CD28 and 4-1BB. A second antigen is selected so that expression of both the first antigen and the second antigen is restricted to the targeted cells (e.g., cancerous tissue or cancerous cells). Similar to a CAR, the extracellular antigen-binding domain can be a scFv, a Fab, a F(ab)₂, or a fusion protein with a heterologous sequence to form the extracellular antigen-binding domain.

In certain embodiments, the CCR is co-expressed with a HI-TCR binding to an antigen that is different from the antigen to which the CCR binds, e.g., the HI-TCR binds to a first antigen and the CCR binds to a second antigen.

Types of human lymphocytes of the presently disclosed subject matter include, without limitation, peripheral donor lymphocytes, e.g., those disclosed in Sadelain, M., *et al.* 2003 *Nat Rev Cancer* 3:35-45 (disclosing peripheral donor lymphocytes genetically modified to express CARs), in Morgan, R.A., *et al.* 2006 *Science* 314:126-129 (disclosing peripheral donor lymphocytes genetically modified to express a full-length tumor antigen-recognizing T cell receptor complex comprising the α and β heterodimer), in Panelli, M.C., *et al.* 2000 *J Immunol* 164:495-504; Panelli, M.C., *et al.* 2000 *J Immunol* 164:4382-4392 (disclosing lymphocyte cultures derived from tumor infiltrating lymphocytes (TILs) in tumor biopsies), and in Dupont, J., *et al.* 2005 *Cancer Res*

65:5417-5427; Papanicolaou, G.A., *et al.* 2003 *Blood* 102:2498-2505 (disclosing selectively *in vitro*-expanded antigen-specific peripheral blood leukocytes employing artificial antigen-presenting cells (AAPCs) or pulsed dendritic cells). The immunoresponsive cells (*e.g.*, T cells) can be autologous, non-autologous (*e.g.*,
5 allogeneic), or derived *in vitro* from engineered progenitor or stem cells.

The presently disclosed immunoresponsive cells are capable of modulating the tumor microenvironment. Tumors have a microenvironment that is hostile to the host immune response involving a series of mechanisms by malignant cells to protect themselves from immune recognition and elimination. This “hostile tumor
10 microenvironment” comprises a variety of immune suppressive factors including infiltrating regulatory CD4⁺ T cells (Tregs), myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), immune suppressive cytokines including TGF-β, and expression of ligands targeted to immune suppressive receptors expressed by activated T cells (CTLA-4 and PD-1). These mechanisms of immune suppression play a
15 role in the maintenance of tolerance and suppressing inappropriate immune responses, however within the tumor microenvironment these mechanisms prevent an effective anti-tumor immune response. Collectively these immune suppressive factors can induce either marked anergy or apoptosis of adoptively transferred CAR modified T cells upon encounter with targeted tumor cells.

20 The unpurified source of CTLs may be any known in the art, such as the bone marrow, fetal, neonate or adult or other hematopoietic cell source, *e.g.*, fetal liver, peripheral blood or umbilical cord blood. Various techniques can be employed to separate the cells. For instance, negative selection methods can remove non-CTLs initially. mAbs are particularly useful for identifying markers associated with particular cell lineages
25 and/or stages of differentiation for both positive and negative selections.

A large proportion of terminally differentiated cells can be initially removed by a relatively crude separation. For example, magnetic bead separations can be used initially to remove large numbers of irrelevant cells. In certain embodiments, at least about 80%, usually at least about 70% of the total hematopoietic cells will be removed prior to cell
30 isolation.

Procedures for separation include, but are not limited to, density gradient centrifugation; resetting; coupling to particles that modify cell density; magnetic separation with antibody-coated magnetic beads; affinity chromatography; cytotoxic agents joined to or used in conjunction with a mAb, including, but not limited to,

complement and cytotoxins; and panning with antibody attached to a solid matrix, e.g. plate, chip, elutriation or any other convenient technique.

Techniques for separation and analysis include, but are not limited to, flow cytometry, which can have varying degrees of sophistication, e.g., a plurality of color
5 channels, low angle and obtuse light scattering detecting channels, impedance channels.

The cells can be selected against dead cells, by employing dyes associated with dead cells such as propidium iodide (PI). In certain embodiments, the cells are collected in a medium comprising 2% fetal calf serum (FCS) or 0.2% bovine serum albumin (BSA) or any other suitable, e.g., sterile, isotonic medium.

10 4. Vectors

Genetic modification of an immunoresponsive cell (e.g., a T cell or an NKT cell) can be accomplished by transducing a substantially homogeneous cell composition with a recombinant DNA construct. In certain embodiments, a retroviral vector (either gamma-retroviral or lentiviral) is employed for the introduction of the DNA construct into
15 the cell. For example, a polynucleotide encoding an HI-TCR can be cloned into a retroviral vector and expression can be driven from its endogenous promoter, from the retroviral long terminal repeat, or from a promoter specific for a target cell type of interest. Non-viral vectors may be used as well.

For initial genetic modification of an immunoresponsive cell to include an HI-
20 TCR, a retroviral vector is generally employed for transduction, however any other suitable viral vector or non-viral delivery system can be used. The HI-TCR can be constructed with an auxiliary molecule (e.g., a cytokine) in a single, multicistronic expression cassette, in multiple expression cassettes of a single vector, or in multiple vectors. Examples of elements that create polycistronic expression cassette include, but is
25 not limited to, various viral and non-viral Internal Ribosome Entry Sites (IRES, e.g., FGF-1 IRES, FGF-2 IRES, VEGF IRES, IGF-II IRES, NF- κ B IRES, RUNX1 IRES, p53 IRES, hepatitis A IRES, hepatitis C IRES, pestivirus IRES, aphthovirus IRES, picornavirus IRES, poliovirus IRES and encephalomyocarditis virus IRES) and cleavable linkers (e.g., 2A peptides, e.g., P2A, T2A, E2A and F2A peptides). Combinations of
30 retroviral vector and an appropriate packaging line are also suitable, where the capsid proteins will be functional for infecting human cells. Various amphotropic virus-producing cell lines are known, including, but not limited to, PA12 (Miller, *et al.* (1985) *Mol. Cell. Biol.* 5:431-437); PA317 (Miller, *et al.* (1986) *Mol. Cell. Biol.* 6:2895-2902); and CRIP (Danos, *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464). Non-

amphotropic particles are suitable too, e.g., particles pseudotyped with VSVG, RD114 or GALV envelope and any other known in the art.

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

Other transducing viral vectors can be used to modify an immunoresponsive cell. In certain embodiments, the chosen vector exhibits high efficiency of infection and stable
 10 integration and expression (see, e.g., Cayouette *et al.*, *Human Gene Therapy* 8:423-430, 1997; Kido *et al.*, *Current Eye Research* 15:833-844, 1996; Bloomer *et al.*, *Journal of Virology* 71:6641-6649, 1997; Naldini *et al.*, *Science* 272:263-267, 1996; and Miyoshi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:10319, 1997). Other viral vectors that can be used include, for example, adenoviral, lentiviral, and adena-associated viral vectors, vaccinia
 15 virus, a bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus (also see, for example, the vectors of Miller, *Human Gene Therapy* 15-14, 1990; Friedman, *Science* 244:1275-1281, 1989; Eglitis *et al.*, *BioTechniques* 6:608-614, 1988; Tolstoshev *et al.*, *Current Opinion in Biotechnology* 1:55-61, 1990; Sharp, *The Lancet* 337:1277-1278, 1991; Cornetta *et al.*, *Nucleic Acid Research and Molecular Biology* 36:311-322, 1987;
 20 Anderson, *Science* 226:401-409, 1984; Moen, *Blood Cells* 17:407-416, 1991; Miller *et al.*, *Biotechnology* 7:980-990, 1989; LeGal La Salle *et al.*, *Science* 259:988-990, 1993; and Johnson, *Chest* 107:77S- 83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg *et al.*, *N. Engl. J. Med* 323:370, 1990; Anderson *et al.*, U.S. Pat. No. 5,399,346).

25 Non-viral approaches can also be employed for genetic modification of an immunoresponsive cell. For example, a nucleic acid molecule can be introduced into an immunoresponsive cell by administering the nucleic acid in the presence of lipofection (Feigner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84:7413, 1987; Ono *et al.*, *Neuroscience Letters* 17:259, 1990; Brigham *et al.*, *Am. J. Med. Sci.* 298:278, 1989; Staubinger *et al.*,
 30 *Methods in Enzymology* 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu *et al.*, *Journal of Biological Chemistry* 263:14621, 1988; Wu *et al.*, *Journal of Biological Chemistry* 264:16985, 1989), or by micro-injection under surgical conditions (Wolff *et al.*, *Science* 247:1465, 1990). Other non-viral means for gene transfer include transfection *in vitro* using calcium phosphate, DEAE dextran, electroporation, and

protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell. Transplantation of normal genes into the affected tissues of a subject can also be accomplished by transferring a normal nucleic acid into a cultivatable cell type *ex vivo* (e.g., an autologous or heterologous primary cell or progeny thereof), after which the cell
5 (or its descendants) are injected into a targeted tissue or are injected systemically.

Recombinant receptors can also be derived or obtained using transposases or targeted nucleases (e.g. Zinc finger nucleases, meganucleases, or TALE nucleases, CRISPR).

Transient expression may be obtained by RNA electroporation. In certain embodiments, recombinant receptors can be introduced by a transposon-based vector. In certain
10 embodiments, the transposon-based vector comprises a transposon (a.k.a. a transposable element). In certain embodiments, the transposon can be recognized by a transposase. In certain embodiments, the transposase is a Sleeping Beauty transposase.

Clustered regularly-interspaced short palindromic repeats (CRISPR) system is a genome editing tool discovered in prokaryotic cells. When utilized for genome editing,
15 the system includes Cas9 (a protein able to modify DNA utilizing crRNA as its guide), CRISPR RNA (crRNA, contains the RNA used by Cas9 to guide it to the correct section of host DNA along with a region that binds to tracrRNA (generally in a hairpin loop form) forming an active complex with Cas9), trans-activating crRNA (tracrRNA, binds to crRNA and forms an active complex with Cas9), and an optional section of DNA repair
20 template (DNA that guides the cellular repair process allowing insertion of a specific DNA sequence). CRISPR/Cas9 often employs a plasmid to transfect the target cells. The crRNA needs to be designed for each application as this is the sequence that Cas9 uses to identify and directly bind to the target DNA in a cell. The repair template carrying CAR expression cassette need also be designed for each application, as it must overlap with the
25 sequences on either side of the cut and code for the insertion sequence. Multiple crRNA's and the tracrRNA can be packaged together to form a single-guide RNA (sgRNA). This sgRNA can be joined together with the Cas9 gene and made into a plasmid in order to be transfected into cells.

A zinc-finger nuclease (ZFN) is an artificial restriction enzyme, which is
30 generated by combining a zinc finger DNA-binding domain with a DNA-cleavage domain. A zinc finger domain can be engineered to target specific DNA sequences which allows a zinc-finger nuclease to target desired sequences within genomes. The DNA-binding domains of individual ZFNs typically contain a plurality of individual zinc finger repeats and can each recognize a plurality of basepairs. The most common method to

generate new zinc-finger domain is to combine smaller zinc-finger "modules" of known specificity. The most common cleavage domain in ZFNs is the non-specific cleavage domain from the type II restriction endonuclease FokI. Using the endogenous homologous recombination (HR) machinery and a homologous DNA template carrying CAR expression cassette, ZFNs can be used to insert the CAR expression cassette into genome. When the targeted sequence is cleaved by ZFNs, the HR machinery searches for homology between the damaged chromosome and the homologous DNA template, and then copies the sequence of the template between the two broken ends of the chromosome, whereby the homologous DNA template is integrated into the genome.

Transcription activator-like effector nucleases (TALEN) are restriction enzymes that can be engineered to cut specific sequences of DNA. TALEN system operates on almost the same principle as ZFNs. They are generated by combining a transcription activator-like effectors DNA-binding domain with a DNA cleavage domain. Transcription activator-like effectors (TALEs) are composed of 33-34 amino acid repeating motifs with two variable positions that have a strong recognition for specific nucleotides. By assembling arrays of these TALEs, the TALE DNA-binding domain can be engineered to bind desired DNA sequence, and thereby guide the nuclease to cut at specific locations in genome. cDNA expression for use in polynucleotide therapy methods can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element or intron (e.g. the elongation factor 1a enhancer/promoter/intron structure). For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct the expression of a nucleic acid. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers. Alternatively, if a genomic clone is used as a therapeutic construct, regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

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

6. Genome Editing Methods

In certain non-limiting embodiments, an HI-TCR, a costimulatory ligand, a CCR or any other molecule/transgene disclosed herein is expressed by an immunoresponsive cell through a modified genomic locus. In certain embodiments, an expression cassette of

the transgene is integrated into a targeted genomic locus of an immunoresponsive cell through targeted genome editing methods. In certain embodiments, the targeted genomic locus can be *CD3δ*, *CD3ε*, *CD247*, *B2M*, *TRAC*, *TRBC1*, *TRBC2*, *TRGC1* and/or *TRGC2* loci.

5 6.1. Engineering T Cell Receptor Locus

In certain embodiments, an HI-TCR is expressed by an immunoresponsive cell through a modified endogenous T cell receptor locus. In certain embodiments, an HI-TCR expression cassette is integrated at an endogenous T cell receptor locus. In certain
10 embodiments, the HI-TCR expression cassette is integrated within the T cell receptor alpha locus (*TRA*, GenBank ID: 6955). In certain embodiments, the HI-TCR expression cassette is integrated within the T cell receptor beta locus (*TRB*, GenBank ID: 6957). In certain embodiments, the HI-TCR expression cassette is integrated within the T cell receptor gamma locus (*TRG*, GenBank ID: 6965).

In certain embodiments, the HI-TCR expression cassette comprises an
15 extracellular antigen-binding domain that is integrated in the first exon of a TCR constant domain locus, so that the extracellular antigen-binding domain and the TCR constant domain are comprised in one antigen binding chain of the HI-TCR. In certain embodiments, the TCR constant domain locus can be *TRAC*, *TRBC1*, *TRBC2*, *TRGC1*, or *TRGC2*. In certain embodiments, the HI-TCR expression cassette comprises an
20 extracellular antigen-binding domain that is integrated in the first exon of a *TRAC* locus, so that the extracellular antigen-binding domain and a *TRAC* peptide are comprised in a first antigen binding chain of the HI-TCR. In certain embodiments, the HI-TCR expression cassette further comprises a second antigen binding chain, which optionally comprises an extracellular antigen-binding domain and a *TRBC* peptide. In certain
25 embodiments, the HI-TCR expression cassette comprises an extracellular antigen-binding domain that is integrated in the first exon of a *TRBC* locus, so that the extracellular antigen-binding domain and a *TRBC* peptide are comprised in a first antigen binding chain of the HI-TCR. In certain embodiments, the HI-TCR expression cassette further comprises a second antigen binding chain, which optionally comprises an extracellular
30 antigen-binding domain and a *TRAC* peptide. In certain embodiments, the expression cassette comprises elements that create polycistronic expression cassette, e.g., a cleavable peptide, e.g., a 2A peptide.

In certain embodiments, the recombinant TCR is expressed from an expression cassette placed in an endogenous *TRAC* locus and/or a *TRBC* locus of an

immunoresponsive cell. In certain embodiments, the placement of the recombinant TCR expression cassette disrupts or abolishes the endogenous expression of a TCR comprising a native TCR α chain and/or a native TCR β chain in the immunoresponsive cell. In certain embodiments, the placement of the recombinant TCR expression cassette prevents or eliminates mispairing between the recombinant TCR and a native TCR α chain and/or a native TCR β chain in the immunoresponsive cell.

Any suitable genetic editing methods and systems can be used to modify an endogenous T cell receptor locus. The genome editing methods disclosed in Section 4 can be used to modify the endogenous T cell receptor locus. In certain embodiments, a CRISPR system is used to modify T cell receptor locus. In certain embodiments, the CRISPR system targets exon 1 of a human TRAC locus. In certain embodiments, the CRISPR system comprises a guide RNA (gRNA) that targets exon 1 of a human TRAC locus.

In certain embodiments, a zinc-finger nuclease is used to modify an endogenous T cell receptor locus. In certain embodiments, a TALEN system is used to modify an endogenous T cell receptor locus.

In certain embodiments, when one endogenous T cell receptor locus in a cell is modified to express one or more antigen binding chain of an HI-TCR, one or more other endogenous T cell receptor locus in the cell are modified to eliminate the endogenous expression of the endogenous TCR chain. In certain embodiments, the one or more other endogenous T cell receptor locus are further modified to express a gene of interest, e.g., an anti-tumor cytokine (e.g., IL-2, IL-12, TNF α , and INF γ), a co-stimulatory molecule ligand (e.g., 4-1BBL), a tracking gene (e.g., eGFP) or a suicide gene.

6.2. Modifying Gene Expression through Genome Editing of a Promoter or a Transcription Terminator

In certain non-limiting embodiments, the expression of an HI-TCR expression cassette integrated into a targeted genomic locus is driven by an endogenous promoter/enhancer of the genomic locus. In certain embodiments, the expression of an HI-TCR expression cassette integrated into a targeted genomic locus is driven by a modified promoter/enhancer introduced to the genomic locus. Any targeted genome editing methods can be used to modify the promoter/enhancer region of a targeted genomic locus, and thereby enhancing or modifying the expression of an HI-TCR in an immunoresponsive cell. In certain embodiments, the modification comprises replacement of an endogenous promoter with a constitutive promoter or an inducible promoter, or

insertion of a constitutive promoter or inducible promoter to the promoter region of a targeted genomic locus. In certain embodiments, a constitutive promoter is positioned on a targeted genomic locus to drive gene expression of the HI-TCR. Eligible constitutive promoters include, but are not limited to, a CMV promoter, an EF1a promoter, a SV40 promoter, a PGK1 promoter, a Ubc promoter, a beta-actin promoter, and a CAG promoter. Alternatively or additionally, a conditional or inducible promoter is positioned on a targeted genomic locus to drive gene expression of the HI-TCR. Non-limiting examples of conditional promoters include a tetracycline response element (TRE) promoter and an estrogen response element (ERE) promoter. In addition, enhancer elements can be placed in regions other than the promoter region.

In certain non-limiting embodiments, the expression of an HI-TCR expression cassette integrated into a targeted genomic locus is regulated by an endogenous transcription terminator of the genomic locus. In certain embodiments, the expression of an HI-TCR expression cassette integrated into a targeted genomic locus is regulated by a modified transcription terminator introduced to the genomic locus. Any targeted genome editing methods can be used to modify the transcription terminator region of a targeted genomic locus, and thereby modifying the expression of an HI-TCR in an immunoresponsive cell. In certain embodiments, the modification comprises replacement of an endogenous transcription terminator with an alternative transcription terminator, or insertion of an alternative transcription terminator to the transcription terminator region of a targeted genomic locus. In certain embodiments, the alternative transcription terminator comprises a 3'UTR region or a ploy A region of a gene. In certain embodiments, the alternative transcription terminator is endogenous. In certain embodiments, the alternative transcription terminator is exogenous. In certain embodiments, alternative transcription terminators include, but are not limited to, a TK transcription terminator, a GCSF transcription terminator, a TCRA transcription terminator, an HBB transcription terminator, a bovine growth hormone transcription terminator, an SV40 transcription terminator and a P2A element.

Any targeted genome editing methods can be used to modify the promoter/enhancer region and/or the transcription terminator region of a targeted genomic locus. In certain embodiments, a CRISPR system is used to modify the promoter/enhancer region and/or the transcription terminator region of a targeted genomic locus. In certain embodiments, zinc-finger nucleases are used to modify the promoter/enhancer region and/or the transcription terminator region of a targeted genomic

locus. In certain embodiments, a TALEN system is used to modify the promoter/enhancer region and/or the transcription terminator region of a targeted genomic locus.

Methods for delivering the genome editing agents/systems can vary depending on the need. In certain embodiments, the components of a selected genome editing method are delivered as DNA constructs in one or more plasmids. In certain embodiments, the components are delivered via viral vectors. Common delivery methods include but is not limited to, electroporation, microinjection, gene gun, impalefection, hydrostatic pressure, continuous infusion, sonication, magnetofection, adeno-associated viruses, envelope protein pseudotyping of viral vectors, replication-competent vectors cis and trans-acting elements, herpes simplex virus, and chemical vehicles (e.g., oligonucleotides, lipoplexes, polymersomes, polyplexes, dendrimers, inorganic Nanoparticles, and cell-penetrating peptides).

Modification can be made anywhere within a targeted genomic locus, or anywhere that can impact gene expression of a targeted genomic locus. In certain embodiments, the modification occurs upstream of the transcriptional start site of a targeted genomic locus. In certain embodiments, the modification occurs between the transcriptional start site and the protein coding region of a targeted genomic locus. In certain embodiments, the modification occurs downstream of the protein coding region of a targeted genomic locus. In certain embodiments, the modification occurs upstream of the transcriptional start site of a targeted genomic locus, wherein the modification produces a new transcriptional start site.

7. Polypeptides and Analogs

Also included in the presently disclosed subject matter are polypeptides (e.g., CD19, CD8, CD28, CD3 ζ , CD40, 4-1BB, OX40, CD84, CD166, CD8a, CD8b, ICOS, ICAM-1, TRAC, TRBC1, TRBC2, TRGC1, TRGC2, CD3 γ , CD3 δ , CD3 ϵ and CTLA-4) or fragments thereof that are modified in ways that enhance their anti-neoplastic activity when expressed in an immunoresponsive cell. The presently disclosed subject matter provides methods for optimizing an amino acid sequence or nucleic acid sequence by producing an alteration in the sequence. Such alterations may include certain mutations, deletions, insertions, or post-translational modifications. The presently disclosed subject matter further includes analogs of any naturally-occurring polypeptide disclosed herein (including, but not limited to, CD19, CD8, CD28, CD3 ζ , CD40, 4-1BB, OX40, CD84, CD166, CD8a, CD8b, ICOS, ICAM-1, TRAC, TRBC1, TRBC2, TRGC1, TRGC2, CD3 γ , CD3 δ , CD3 ϵ and CTLA-4). Analogs can differ from a naturally-occurring

polypeptide disclosed herein by amino acid sequence differences, by post-translational modifications, or by both. Analogs can exhibit at least about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or more homologous to all or part of a naturally-occurring amino, acid
5 sequence of the presently disclosed subject matter. The length of sequence comparison is at least 5, 10, 15 or 20 amino acid residues, e.g., at least 25, 50, or 75 amino acid residues, or more than 100 amino acid residues. Again, in an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence. Modifications include *in vivo* and *in*
10 *vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring polypeptides by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting
15 from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989, or Ausubel et al., supra). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ
20 amino acids.

In addition to full-length polypeptides, the presently disclosed subject matter also provides fragments of any one of the polypeptides or peptide domains disclosed herein. As used herein, the term "a fragment" means at least 5, 10, 13, or 15 amino acids. In certain embodiments, a fragment comprises at least 20 contiguous amino acids, at least 30
25 contiguous amino acids, or at least 50 contiguous amino acids. In certain embodiments, a fragment comprises at least 60 to 80, 100, 200, 300 or more contiguous amino acids. Fragments can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by
30 alternative mRNA splicing or alternative protein processing events).

Non-protein analogs have a chemical structure designed to mimic the functional activity of a protein disclosed herein. Such analogs may exceed the physiological activity of the original polypeptide. Methods of analog design are well known in the art, and

synthesis of analogs can be carried out according to such methods by modifying the chemical structures such that the resultant analogs increase the anti-neoplastic activity of the original polypeptide when expressed in an immunoresponsive cell. These chemical modifications include, but are not limited to, substituting alternative R groups and varying the degree of saturation at specific carbon atoms of a reference polypeptide. In certain embodiments, the protein analogs are relatively resistant to *in vivo* degradation, resulting in a more prolonged therapeutic effect upon administration. Assays for measuring functional activity include, but are not limited to, those described in the Examples below.

8. Administration

Compositions comprising the presently disclosed immunoresponsive cells can be provided systemically or directly to a subject for inducing and/or enhancing an immune response to an antigen and/or treating and/or preventing a neoplasia, pathogen infection, or infectious disease. In certain embodiments, the presently disclosed immunoresponsive cells or compositions comprising thereof are directly injected into an organ of interest (e.g., an organ affected by a neoplasia). Alternatively, the presently disclosed immunoresponsive cells or compositions comprising thereof are provided indirectly to the organ of interest, for example, by administration into the circulatory system (e.g., the tumor vasculature). Expansion and differentiation agents can be provided prior to, during or after administration of the cells or compositions to increase production of T cells, NKT cells, or CTL cells *in vitro* or *in vivo*.

The presently disclosed immunoresponsive cells can be administered in any physiologically acceptable vehicle, normally intravascularly, although they may also be introduced into bone or other convenient site where the cells may find an appropriate site for regeneration and differentiation (e.g., thymus). Usually, at least about 1×10^5 cells will be administered, eventually reaching about 1×10^{10} or more. The presently disclosed immunoresponsive cells can comprise a purified population of cells. Those skilled in the art can readily determine the percentage of the presently disclosed immunoresponsive cells in a population using various well-known methods, such as fluorescence activated cell sorting (FACS). Suitable ranges of purity in populations comprising the presently disclosed immunoresponsive cells are about 50% to about 55%, about 5% to about 60%, and about 65% to about 70%. In certain embodiments, the purity is about 70% to about 75%, about 75% to about 80%, or about 80% to about 85%. In certain embodiments, the purity is about 85% to about 90%, about 90% to about 95%, and about 95% to about 100%. Dosages can be readily adjusted by those skilled in the art (e.g., a decrease in

purity may require an increase in dosage). The cells can be introduced by injection, catheter, or the like.

The presently disclosed compositions can be pharmaceutical compositions comprising the presently disclosed immunoresponsive cells or their progenitors and a pharmaceutically acceptable carrier. Administration can be autologous or heterologous. For example, immunoresponsive cells, or progenitors can be obtained from one subject, and administered to the same subject or a different, compatible subject. Peripheral blood derived immunoresponsive cells or their progeny (e.g., *in vivo*, *ex vivo* or *in vitro* derived) can be administered via localized injection, including catheter administration, systemic injection, localized injection, intravenous injection, or parenteral administration. When administering a therapeutic composition of the presently disclosed subject matter (e.g., a pharmaceutical composition comprising a presently disclosed immunoresponsive cell), it can be formulated in a unit dosage injectable form (solution, suspension, emulsion).

9. Formulations

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

Sterile injectable solutions can be prepared by incorporating the genetically modified immunoresponsive cells in the required amount of the appropriate solvent with various amounts of the other ingredients, as desired. Such compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting, dispersing, or emulsifying agents (e.g., methylcellulose), pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as “REMINGTON’S

PHARMACEUTICAL SCIENCE”, 17th edition, 1985, incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation.

Various additives which enhance the stability and sterility of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the presently disclosed subject matter, however, any vehicle, diluent, or additive used would have to be compatible with the genetically modified immunoresponsive cells or their progenitors.

The compositions can be isotonic, i.e., they can have the same osmotic pressure as blood and lacrimal fluid. The desired isotonicity of the compositions may be accomplished using sodium chloride, or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol or other inorganic or organic solutes. Sodium chloride can be particularly for buffers containing sodium ions.

Viscosity of the compositions, if desired, can be maintained at the selected level using a pharmaceutically acceptable thickening agent. For example, methylcellulose is readily and economically available and is easy to work with. Other suitable thickening agents include, for example, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carbomer, and the like. The concentration of the thickener can depend upon the agent selected. The important point is to use an amount that will achieve the selected viscosity. Obviously, the choice of suitable carriers and other additives will depend on the exact route of administration and the nature of the particular dosage form, e.g., liquid dosage form (e.g., whether the composition is to be formulated into a solution, a suspension, gel or another liquid form, such as a time release form or liquid-filled form).

The quantity of cells to be administered will vary for the subject being treated. In a one embodiment, between about 10^4 and about 10^{10} , between about 10^5 and about 10^9 , or between about 10^6 and about 10^8 of the presently disclosed immunoresponsive cells are administered to a human subject. More effective cells may be administered in even smaller numbers. In certain embodiments, at least about 1×10^8 , about 2×10^8 , about 3×10^8 , about 4×10^8 , or about 5×10^8 of the presently disclosed immunoresponsive cells are administered to a human subject. The precise determination of what would be considered an effective dose may be based on factors individual to each subject, including their size,

age, sex, weight, and condition of the particular subject. Dosages can be readily ascertained by those skilled in the art from this disclosure and the knowledge in the art.

The skilled artisan can readily determine the amount of cells and optional additives, vehicles, and/or carrier in compositions and to be administered in methods.

- 5 Typically, any additives (in addition to the active cell(s) and/or agent(s)) are present in an amount of 0.001 to 50% (weight) solution in phosphate buffered saline, and the active ingredient is present in the order of micrograms to milligrams, such as about 0.0001 to about 5 wt %, about 0.0001 to about 1 wt %, about 0.0001 to about 0.05 wt% or about 0.001 to about 20 wt %, about 0.01 to about 10 wt %, or about 0.05 to about 5 wt %. For
10 any composition to be administered to an animal or human, the followings can be determined: toxicity such as by determining the lethal dose (LD) and LD50 in a suitable animal model e.g., rodent such as mouse; the dosage of the composition(s), concentration of components therein and timing of administering the composition(s), which elicit a suitable response. Such determinations do not require undue experimentation from the
15 knowledge of the skilled artisan, this disclosure and the documents cited herein. And, the time for sequential administrations can be ascertained without undue experimentation.

10. Methods of Treatment

- The presently disclosed subject matter provides methods for inducing and/or increasing an immune response in a subject in need thereof. The presently disclosed
20 immunoresponse cells and compositions comprising thereof can be used for treating and/or preventing a neoplasia in a subject. The presently disclosed immunoresponse cells and compositions comprising thereof can be used for prolonging the survival of a subject suffering from a neoplasia. The presently disclosed immunoresponse cells and compositions comprising thereof can also be used for treating and/or preventing a
25 pathogen infection or other infectious disease in a subject, such as an immunocompromised human subject. Such methods comprise administering the presently disclosed immunoresponse cells in an amount effective or a composition (e.g., pharmaceutical composition) comprising thereof to achieve the desired effect, be it palliation of an existing condition or prevention of recurrence. For treatment, the amount
30 administered is an amount effective in producing the desired effect. An effective amount can be provided in one or a series of administrations. An effective amount can be provided in a bolus or by continuous perfusion.

In certain embodiments, immunoresponse cells comprising a HI-TCR disclosed herein can be used to treat a subject having tumor cells with a low expression level of a

surface antigen, e.g. from a relapse of a disease, wherein the subject received treatment which leads to residual tumor cells. In certain embodiments, the tumor cells have low density of a target molecule on the surface of the tumor cells. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 5,000 molecules per cell, less than about 4,000 molecules per cell, less than about 3,000 molecules per cell, less than about 2,000 molecules per cell, less than about 1,500 molecules per cell, less than about 1,000 molecules per cell, less than about 500 molecules per cell, less than about 200 molecules per cell, or less than about 100 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 2,000 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 1,500 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of less than about 1,000 molecules per cell. In certain embodiments, a target molecule having a low density on the cell surface has a density of between about 4,000 molecules per cell and about 2,000 molecules per cell, between about 2,000 molecules per cell and about 1,000 molecules per cell, between about 1,500 molecules per cell and about 1,000 molecules per cell, between about 2,000 molecules per cell and about 500 molecules per cell, between about 1,000 molecules per cell and about 200 molecules per cell, or between about 1,000 molecules per cell and about 100 molecules per cell. In certain embodiments, immunoresponsive cells comprising a HI-TCR disclosed herein can be used to treat a subject having a relapse of a disease, wherein the subject received immunoresponsive cells (e.g., T cells) comprising a CAR comprising an intracellular signaling domain that comprises a co-stimulatory signaling domain comprising a 4-1BB polypeptide (e.g., a 4-1BBz CAR). In certain embodiments, the tumor cells have a low density of a tumor specific antigen on the surface of the tumor cells. In certain embodiments, the disease is CD19⁺ ALL. In certain embodiments, the tumor cells have a low density of CD19 on the tumor cells. Such methods comprise administering the presently disclosed immunoresponsive cells in an amount effective or a composition (e.g., pharmaceutical composition) comprising thereof to achieve the desired effect, alleviation of an existing condition or prevention of recurrence.

An “effective amount” (or, “therapeutically effective amount”) is an amount sufficient to effect a beneficial or desired clinical result upon treatment. An effective amount can be administered to a subject in one or more doses. In terms of treatment, an

effective amount is an amount that is sufficient to palliate, ameliorate, stabilize, reverse or slow the progression of the disease, or otherwise reduce the pathological consequences of the disease. The effective amount is generally determined by the physician on a case-by-case basis and is within the skill of one in the art. Several factors are typically taken into account when determining an appropriate dosage to achieve an effective amount. These factors include age, sex and weight of the subject, the condition being treated, the severity of the condition and the form and effective concentration of the immunoresponsive cells administered.

For adoptive immunotherapy using antigen-specific T cells, cell doses in the range of about 10^6 - 10^{10} (e.g., about 10^9) are typically infused. Upon administration of the presently disclosed cells into the host and subsequent differentiation, T cells are induced that are specifically directed against the specific antigen. The modified cells can be administered by any method known in the art including, but not limited to, intravenous, subcutaneous, intranodal, intratumoral, intrathecal, intrapleural, intraperitoneal and directly to the thymus.

The presently disclosed subject matter provides methods for treating and/or preventing a neoplasia in a subject. The method can comprise administering an effective amount of the presently disclosed immunoresponsive cells or a composition comprising thereof to a subject having a neoplasia.

Non-limiting examples of neoplasia include blood cancers (e.g. leukemias, lymphomas, and myelomas), ovarian cancer, breast cancer, bladder cancer, brain cancer, colon cancer, intestinal cancer, liver cancer, lung cancer, pancreatic cancer, prostate cancer, skin cancer, stomach cancer, glioblastoma, throat cancer, melanoma, neuroblastoma, adenocarcinoma, glioma, soft tissue sarcoma, and various carcinomas (including prostate and small cell lung cancer). Suitable carcinomas further include any known in the field of oncology, including, but not limited to, astrocytoma, fibrosarcoma, myxosarcoma, liposarcoma, oligodendroglioma, ependymoma, medulloblastoma, primitive neural ectodermal tumor (PNET), chondrosarcoma, osteogenic sarcoma, pancreatic ductal adenocarcinoma, small and large cell lung adenocarcinomas, chordoma, angiosarcoma, endotheliosarcoma, squamous cell carcinoma, bronchoalveolarcarcinoma, epithelial adenocarcinoma, and liver metastases thereof, lymphangiosarcoma, lymphangioendotheliosarcoma, hepatoma, cholangiocarcinoma, synovioma, mesothelioma, Ewing's tumor, rhabdomyosarcoma, colon carcinoma, basal cell carcinoma, sweat gland carcinoma, papillary carcinoma, sebaceous gland carcinoma,

- papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, testicular tumor, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma,
- 5 oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, leukemia, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease, breast tumors such as ductal and lobular adenocarcinoma, squamous and adenocarcinomas of the uterine cervix, uterine and ovarian epithelial carcinomas, prostatic adenocarcinomas, transitional squamous cell carcinoma of the bladder, B and T cell lymphomas (nodular
- 10 and diffuse) plasmacytoma, acute and chronic leukemias, malignant melanoma, soft tissue sarcomas and leiomyosarcomas. In certain embodiments, the neoplasm is selected from the group consisting of blood cancers (e.g. leukemias, lymphomas, and myelomas), ovarian cancer, prostate cancer, breast cancer, bladder cancer, brain cancer, colon cancer, intestinal cancer, liver cancer, lung cancer, pancreatic cancer, prostate cancer, skin
- 15 cancer, stomach cancer, glioblastoma, and throat cancer. In certain embodiments, the presently disclosed immunoresponsive cells and compositions comprising thereof can be used for treating and/or preventing blood cancers (e.g., leukemias, lymphomas, and myelomas) or ovarian cancer, which are not amenable to conventional therapeutic interventions. In certain embodiments, the neoplasm is a solid tumor.
- 20 The subjects can have an advanced form of disease, in which case the treatment objective can include mitigation or reversal of disease progression, and/or amelioration of side effects. The subjects can have a history of the condition, for which they have already been treated, in which case the therapeutic objective will typically include a decrease or delay in the risk of recurrence.
- 25 Suitable human subjects for therapy typically comprise two treatment groups that can be distinguished by clinical criteria. Subjects with "advanced disease" or "high tumor burden" are those who bear a clinically measurable tumor. A clinically measurable tumor is one that can be detected on the basis of tumor mass (e.g., by palpation, CAT scan, sonogram, mammogram or X-ray; positive biochemical or histopathologic markers on
- 30 their own are insufficient to identify this population). A pharmaceutical composition is administered to these subjects to elicit an anti-tumor response, with the objective of palliating their condition. Ideally, reduction in tumor mass occurs as a result, but any clinical improvement constitutes a benefit. Clinical improvement includes decreased risk or rate of progression or reduction in pathological consequences of the tumor.

A second group of suitable subjects is known in the art as the “adjuvant group.” These are individuals who have had a history of neoplasia, but have been responsive to another mode of therapy. The prior therapy can have included, but is not restricted to, surgical resection, radiotherapy, and traditional chemotherapy. As a result, these
5 individuals have no clinically measurable tumor. However, they are suspected of being at risk for progression of the disease, either near the original tumor site, or by metastases. This group can be further subdivided into high-risk and low-risk individuals. The subdivision is made on the basis of features observed before or after the initial treatment. These features are known in the clinical arts, and are suitably defined for each different
10 neoplasia. Features typical of high-risk subgroups are those in which the tumor has invaded neighboring tissues, or who show involvement of lymph nodes.

Another group have a genetic predisposition to neoplasia but have not yet evidenced clinical signs of neoplasia. For instance, women testing positive for a genetic mutation associated with breast cancer, but still of childbearing age, can wish to receive
15 one or more of the immunoresponsive cells described herein in treatment prophylactically to prevent the occurrence of neoplasia until it is suitable to perform preventive surgery.

Additionally, the presently disclosed subject matter provides methods for treating and/or preventing a pathogen infection (e.g., viral infection, bacterial infection, fungal infection, parasite infection, or protozoal infection) in a subject, e.g., in an
20 immunocompromised subject. The method can comprise administering an effective amount of the presently disclosed immunoresponsive cells or a composition comprising thereof to a subject having a pathogen infection. Exemplary viral infections susceptible to treatment include, but are not limited to, Cytomegalovirus (CMV), Epstein Barr Virus (EBV), Human Immunodeficiency Virus (HIV), and influenza virus infections.

Further modification can be introduced to the presently disclosed
25 immunoresponsive cells (e.g., T cells) to avert or minimize the risks of immunological complications (known as “malignant T-cell transformation”), e.g., graft versus-host disease (GvHD), or when healthy tissues express the same target antigens as the tumor cells, leading to outcomes similar to GvHD. A potential solution to this problem is
30 engineering a suicide gene into the presently disclosed immunoresponsive cells. Suitable suicide genes include, but are not limited to, Herpes simplex virus thymidine kinase (hsv-tk), inducible Caspase 9 Suicide gene (iCasp-9), and a truncated human epidermal growth factor receptor (EGFRt) polypeptide. In certain embodiments, the suicide gene is an EGFRt polypeptide. The EGFRt polypeptide can enable T cell elimination by

administering anti-EGFR monoclonal antibody (*e.g.*, cetuximab). EGFRt can be covalently joined to the upstream of the antigen-recognizing receptor of a presently disclosed CAR. The suicide gene can be included within the vector comprising nucleic acids encoding a presently disclosed CAR. In this way, administration of a prodrug
5 designed to activate the suicide gene (*e.g.*, a prodrug (*e.g.*, AP1903 that can activate iCasp-9) during malignant T-cell transformation (*e.g.*, GVHD) triggers apoptosis in the suicide gene-activated CAR-expressing T cells. The incorporation of a suicide gene into the a presently disclosed CAR gives an added level of safety with the ability to eliminate the majority of CAR T cells within a very short time period. A presently disclosed
10 immunoresponsive cell (*e.g.*, a T cell) incorporated with a suicide gene can be pre-emptively eliminated at a given timepoint post CAR T cell infusion, or eradicated at the earliest signs of toxicity.

11. Kits

The presently disclosed subject matter provides kits for inducing and/or enhancing
15 an immune response and/or treating and/or preventing a neoplasia or a pathogen infection in a subject. In certain embodiments, the kit comprises an effective amount of presently disclosed immunoresponsive cells or a pharmaceutical composition comprising thereof. In certain embodiments, the kit comprises a sterile container; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable
20 container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments. In certain non-limiting embodiments, the kit includes an isolated nucleic acid molecule encoding an HI-TCR disclosed herein which is directed toward an antigen of interest in expressible form, which may optionally be comprised in one or more vectors.

25 If desired, the immunoresponsive cells and/or nucleic acid molecules are provided together with instructions for administering the cells or nucleic acid molecules to a subject having or at risk of developing a neoplasia or pathogen or immune disorder. The instructions generally include information about the use of the composition for the treatment and/or prevention of neoplasia or a pathogen infection. In certain
30 embodiments, the instructions include at least one of the following: description of the therapeutic agent; dosage schedule and administration for treatment or prevention of a neoplasia, pathogen infection, or immune disorder or symptoms thereof; precautions; warnings; indications; counter-indications; over-dosage information; adverse reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed

directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

EXAMPLES

The practice of the present disclosure employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, “Molecular Cloning: A Laboratory Manual”, second edition (Sambrook, 1989); “Oligonucleotide Synthesis” (Gait, 1984); “Animal Cell Culture” (Freshney, 1987); “Methods in Enzymology” “Handbook of Experimental Immunology” (Weir, 1996); “Gene Transfer Vectors for Mammalian Cells” (Miller and Calos, 1987); “Current Protocols in Molecular Biology” (Ausubel, 1987); “PCR: The Polymerase Chain Reaction”, (Mullis, 1994); “Current Protocols in Immunology” (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides disclosed herein, and, as such, may be considered in making and practicing the the presently disclosed subject matter. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the presently disclosed cells and compositions, and are not intended to limit the scope of what the inventors regard as their invention.

Example 1 - Targeting T cells with endogenous non-HLA restricted T cell receptor/CD3 complexes

Introduction

Specific modifications were made at a TCR locus designed to alter the antigen specificity of human T cells without disrupting or bypassing their CD3 complex, which physiologically controls and regulates T cell activation. In this approach, the T cell lost its endogenous T cell receptor and acquired the ability to recognize antigen independent of HLA, making use of its CD3 complex, which is otherwise bypassed when using CARs. This was accomplished through the targeted disruption of the endogenous TCR combined with the introduction of antigen binding domains into the TRAC locus in such a way as to preserve all components of the natural CD3 complex in their native structure. The recombinant TCR-like molecule (i.e., HI-TCR, FvTCR or HIT-CAR) can harbor variable domains derived from immunoglobulin genes, or alternative ligands, to direct antigen

recognition. The recombined receptor signals via the CD3 complex, which can either be intact (i.e., in its physiological composition) or augmented through the non-covalent incorporation of costimulatory domains.

Results

5 A novel strategy for one-step generation of TCR and TCR-like T cells was developed by integrating a TCR or TCR-like gene with a distinct variable domain for a predetermined antigen. This resulted in the expression of the new TCR/TCR-like gene under the control of the endogenous TCR promoter (alpha, beta or both) with concomitant disruption of surface expression of the endogenous TCR.

10 A strategy for one-step generation of universal CAR T cells were developed by targeting the integration of a promoter-less CAR gene cassette in the TCR alpha constant chain (TRAC) first exon. This resulted in CAR expression under the control of the endogenous TCR alpha promoter with concomitant disruption of the TCR alpha gene expression, leading to lack of TCR expression at the cell surface. CAR gene targeting at
15 the TRAC locus was accomplished through homologous recombination (HR) by using a site-specific nuclease (e.g. CRISPR/Cas9) and an AAV donor template. Using HR-based gene targeting, the present strategy permitted the generation of T cells with a unique specificity, which was encoded by a chimeric TCR receptor containing a specific antigen-binding domain. The antigen-binding domain was derived from either an
20 Immunoglobulin (i.e. Fv fragment), a ligand for a cell-surface receptor or a TCR ($\alpha\beta$ or $\gamma\delta$). The hybrid Immunoglobulin-TCR chimeric antigen receptor (i.e., HIT-CAR, FvTCR or HI-TCR) or the $\gamma\delta$ TCR allowed the T cell to recognize a target cell in an HLA-independent manner. Inserting a new $\alpha\beta$ TCR could be accomplished in the same way but would result in HLA-restricted antigen recognition.

25 For example, Figure 1A shows schematic representation of the T Cell Receptor (TCR), the B Cell Receptor (BCR), a Chimeric Antigen Receptor (CAR) and the HLA-Independent TCR-based Chimeric Antigen Receptor (i.e., HIT-CAR, FvTCR or HI-TCR). CRISPR/Cas9-targeted integration of the three receptors into the TRAC locus is shown in Figure 1B. The engineered HIT-CAR targets CD19. Cell surface expression of
30 HIT-CAR (i.e., HI-TCR) from TRAC locus is shown in Figure 1C. Cytolytic effects and proliferation of HIT-CAR (i.e., HI-TCR or FvTCR) T cells are shown in Figures 1D and 1E respectively.

Conditions were established to yield up to 65% of HIT-CAR (i.e., HI-TCR or FvTCR) T cells combining target gene disruption and HIT-CAR (i.e., HI-TCR or FvTCR)

targeted insertion in a single step as shown in Figure 2A. These T cells exhibited *in vitro* and *in vivo* tumor lysis activity similar to the previously characterized TRAC-CAR T cells expressing the 1928z CAR as shown in Figure 2B. In addition, antigen interaction induced down-regulation of the HIT-CAR (i.e., HI-TCR or FvTCR) T cells, which was dependent of the number of antigen-dependent stimulations. As endogenous TCR expression is eliminated at the cell surface, these HIT-CAR (i.e., HI-TCR or FvTCR) T cells were useful for the development of off-the-shelf immunotherapy.

Targets other than CD19 can be employed for immunotherapy. Schematic representation of the NYESO TCR genes integrated into the TCR alpha or beta chain is shown in Figure 3A. Cell surface expressions of TRAC-NYESO-TCR and TRBC-NYESO-TCR (i.e., HI-TCRs or FvTCRs) are shown in Figure 3B. Representative TCR-V-beta-1 flow cytometry plots 4 days after TRAC or TRBC targeting are shown in Figure 3B. Cytotoxic activity of the engineered T cells is shown in Figure 3C. Schematic representation of an alternative design of co-targeting into both the TCR alpha and the TCR beta is shown in Figure 3D, where a NYESO-HI-TCR was placed in the TRAC locus and 4-1BBL expression cassette was placed in TRBC locus. Cell surface expressions of the TRAC-NYESO-TCR and TRBC-4-1BBL are shown in Figure 3E.

A variant approach was also provided in which a costimulatory domain is non-covalently inserted into the CD3 complex, which provides supraphysiological antigen sensing and activation. This is achieved by fusing the costimulatory domain to one or both TCR chains. Moreover, two costimulatory dosages are possible, by either fusing the costimulatory domain to one or both modified TCR chains (i.e., antigen binding chains).

Moreover, HI-TCR exhibited greater sensitivity in comparison to CARs. Human T cells edited to replace endogenous TCR with HI-TCR acquired the ability to engage lower antigen densities and kill such cells. For example, Table 2 shows *in vitro* cytotoxic activity of HI-TCR cells. HI-TCR or CD19-CAR was introduced at the endogenous TRAC locus of human peripheral T cells via CRISPR/Cas9-mediated gene editing and AAV6 donor vectors. Five days post-gene targeting, T cells (6, 30, or 150 thousands) were incubated with 250 thousands Nalm6 leukemia cells that express different CD19 levels (from very low to high levels). Cells were incubated for 22h in 500-ul of X-Vivo medium containing serum (without IL2). Co-cultures were analyzed by FACS in the presence of counting beads to determine the total number of Nalm6 cells. Cytotoxic activity is shown as a percentage of Nalm6 killed. E/T: effector (T cells) to target (Nalm6) ratio. The data clearly demonstrate that the HI-TCR can detect lower levels of

CD19 antigen than a CAR. This feature can be very useful for antigens with moderate or low expression such as CD22, BCMA, CCR1, CD70, etc., and can also be useful to treat relapse after CAR therapy for any antigen, as the relapse tumor cells frequently show reduced antigen densities on their surface.

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Table 2. Tumor Cell Killing Percentage

E/T	HI-TCR	CD19 CAR	CD19 levels
0.024	0	0	very low
	22.53	16.32	low
	29.19	17.18	medium
	38.3	32	high
0.12	15.48	0.4	very low
	51.46	53.31	low
	54.14	63.58	medium
	63.28	63.38	high
0.6	30.36	8.37	very low
	68.32	77.2	low
	77.01	79.38	medium
	78.72	84.37	high

Furthermore, a panel of exogenous and endogenous 3' untranslated regions (3'UTR) were found as capable of regulate precise and predictable gene expression at the TCR locus. For example, Figure 4A shows schematic representation of the CD28z CAR gene integrated into the TRAC locus. Poly A (black box) corresponds to the segment of the CAR cassette that was modified to test different viral and mammalian 3' UTRs. Using TRAC-CAR T cells expressing the CD28z CAR as a model, certain 3'UTRs were shown to lower the CAR expression compared to the bovine growth hormone (bGH) polyA sequence (Figures 4B and 4C), which yielded CAR-T cells with impaired *in vivo* cytotoxic activity (Figures 5A and 5B). Certain other 3'UTRs, including the endogenous TRAC 3'UTR, were shown to increase CAR surface expression levels (Figures 4B and 4C), which produced CAR-T cells with improved *in vivo* cytotoxic activity (Figures 5A and 5B). These 3'UTRs can also be used to regulate precise and predictable gene expression of any HI-TCR disclosed herein.

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The above-described genetic modifications at the TRAC locus permit the generation of T cells expressing optimal levels of HI-TCR that like the physiological TCR but unlike CARs, take advantage of the endogenous T cell activating machinery (the CD3 complex and downstream signaling elements). This approach can advance both autologous and allogeneic T cell therapies.

Another relevant area where TCR gene editing has relevant applications is T-iPS-derived T cells. T-iPS cells are pluripotent stem cells obtained through reprogramming of peripheral T lymphocytes. These T-iPS cells therefore contain a rearranged T-cell receptor, either an $\alpha\beta$ or a $\gamma\delta$ TCR, which can be modified using gene-editing technologies. T cells obtained from T-iPS cells through directed differentiation express the rearranged TCR, which can be detected and sequenced. Using this sequence, one can determine the precise location of the rearranged variable domain in the genome of T-iPS cells. Using nucleases that specifically target this rearranged variable domain and a donor DNA containing a TCR variable domain of known specificity, one can replace the endogenous variable domain with the new one. This approach requires two steps of gene editing: one targeting the α (or γ) chain, and the other the β (or δ) chain. The approach can be performed in a single step since only two alleles need to be modified. In addition, using a strategy similar as to the one described for primary human T cells, the TCR chains can be modified to express any HI-TCR disclose herein.

20 **Example 2**

CRISPR/Cas9-targeted integration of the three receptors into the TRAC locus is shown in Figure 1A. The engineered HIT-CAR targets CD19. Cell surface expression of HIT-CAR (i.e., HI-TCR) from TRAC locus is show in Figures 6B and 6C. Cytolytic effects and proliferation of HIT-CAR (i.e., HI-TCR or FvTCR) T cells are shown in Figures 7A,7B and 8A-8C. In particular, these data show that HIT T cells outperformed CAR-T cells at killing target cells expressing low antigen levels.

Furthermore, as shown in Figures 9A-9D the HI-TCR T cells were engineered to co-express costimulatory ligands. In this specific experiment, the day after targeting the HI-TCR to the TRAC locus, the T cells were transferred with retroviral SFG vectors coding for CD80, 41BBL or both. 5 days after expanding these cells ex vivo, 4e5 TRAC-CAR or TRAC-HI-TCR positive T cells were injected into NSG mice bearing NALM6 cells expressing very low level of CD19. The TRAC-HI-TCR T cells we either expressing CD80, 41BBL, both ligand or none. Bioluminescence was used to assess the tumor burden every week and follow the survival of the mice.

It was observed that the TRAC-CAR T cells were not able to control the tumor with very low levels of CD19 and all the mice were terminally ill by day 30. At this low dose, the HI-TCR T cells initially controlled the tumor burden, however the mice relapsed by day 10 and were terminally ill by day 40. The addition of costimulatory ligands to the HI-TCR T cells improved the anti-tumor activity with an optimal response when the HI-TCR T cells co-expressed CD80 and 4-1BBL. This improved activity resulted in a long-term control of NALM6 expressing very low levels of CD19.

Figures 10A-10C further demonstrate that baseline TRAC-CAR expression can be controlled by distinct 3'UTR sequences without affecting cell surface replenishment kinetic after antigen encounter. The TRAC-CAR T cells were engineered the same way as previously described, so that the all the constructs were under the transcriptional control of the TRAC endogenous promoter. It was observed that by modifying the 3'UTR (that includes polyA signal), the baseline expression level were modulated(Figure 10B). When these different TRAC-CAR T cells were cultivated on CD19 expressing tumor cells, a drop in the CAR cell surface expression followed by a replenishment was observed by flow cytometry (Figure 10 C). The different 3'UTR changed the baseline expression level but they retained the same replenishment kinetic and the final expression level was similar to the baseline.

Furthermore, a HI-TCR targeting CD70 and a HI-TCR targeting CD22 were also created. HI-TCR targeting an interested antigen can be created by sequencing an existing scFv or a Fab region of an existing antibody targeting the same antigen to obtain the extracellular antigen-binding domain.

Embodiments of the presently disclosed subject matter

From the foregoing description, it will be apparent that variations and modifications may be made to the presently disclosed subject matter to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or sub-combination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.