HUMANIZED ANTI-HLA-G CHIMERIC ANTIGEN RECEPTORS AND USES THEREOF

Cross-reference to Related Applications

[0001] This application claims priority to U.S. Provisional Patent Application No. 63/385,142, filed November 28, 2022, which is incorporated by reference herein in its entirety for all purposes

Reference to an Electronic Sequence Listing

[0002] The contents of the electronic sequence listing (INVE_015_01WO_SeqList_ST26.xml; Size: 84,578 bytes; and Date of Creation: November 27, 2023) are herein incorporated by reference in its entirety.

Field

[0003] The present disclosure relates to the fields of immunology, cell biology, and molecular biology. More specifically, the disclosure relates to humanized chimeric antigen receptors (CARs) that bind human leukocyte antigen (HLA-G), cells expressing such CARs and therapeutic uses of these compositions.

Background

[0004] For years, the foundations of cancer treatment were surgery, chemotherapy, and radiation therapy. More recently, immunotherapy has emerged as an effective tool in cancer treatment.

[0005] Chimeric antigen receptors (CARs) are synthetic tumor targeting receptors that can be introduced into human immune cells such as T cells to redirect antigen specificity and enhance functions of effector immune cells. While many attempts to achieve the success of CAR T cells for solid tumors have been undertaken results have been sometimes disappointing. The three main hurdles encountered for the application of CAR T cell therapies to solid tumors are (1) the identification of proper Tumor Associated Antigens (TAA), (2) the limited trafficking of adoptively transferred cells to tumor sites (3) the immunosuppressive effect of tumor microenvironment and (4) the potential safety issue of unwanted or untransformed cells.

[0006] Human leukocyte antigen G (HLA-G) is a molecule that plays an important role in the generation of fetal-induced maternal immunological tolerance. HLA-G has also been proposed

as an immune checkpoint (ICP) molecule that inhibits the effector functions of infiltrating immune cell subsets through the interaction with its specific receptors. HLA-G is expressed in numerous tumor effusions of diverse origins with a highly restricted tissue expression. In several malignant transformations, the expression of HLA-G by tumor cells rises dramatically, rendering them strongly immunosuppressive. Preclinical models have shown that the expression of HLA-G on cancer cells renders them more metastatic and significantly decreases patient survival (Lin A et al.. Int J Cancer. 2012 Jul 1;131(1):150-7; Lin A et al. Hum Immunol. 2013 Apr;74(4):439-46). Additional immunotherapeutic approaches targeting HLA-G are needed to treat cancer

SUMMARY

[0007] Provided herein is a humanized chimeric antigen receptor (CAR) comprising: (a) an extracellular domain comprising a humanized antigen-binding domain that specifically binds to human leukocyte antigen G (HLA-G); (b) a transmembrane region; and (c) an intracellular domain. Humanized anti-HLA-G CARs as disclosed herein exhibit reduced immunogenicity in human patients.

[0008] In some embodiments, the humanized antigen-binding domain of an anti-HLA-G CAR is a humanized antigen-binding fragment of an anti- HLA-G CAR antibody. In some embodiments, the humanized antigen-binding fragment comprises a humanized heavy chain variable (VH) region comprising the HCDR1, HCDR2 and HCDR3 and a humanized light chain variable (VL) region comprising the LCDR1, LCDR2 and LCDR3 of an anti-HLA-G antibody. In some embodiments, the humanized antigen-binding fragment comprises: the HCDR1 of SEQ ID NO: 11, the HCDR2 of SEQ ID NO: 13, and the HCDR3 of SEQ ID NO: 15; and the LCDR1 of SEQ ID NO: 37, the LCDR2 of SEQ ID NO: 39, the LCDR3 of SEQ ID NO: 41.

[0009] Further provided herein is an anti-HLA-G CAR comprising a humanized antigen-binding fragment that comprises:

(a) (i) a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 25; and (ii) a light chain variable region (VL) comprising the sequence of SEQ ID NO: 35;

(b) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 50; or

(c) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 60.

[0010] Also provided herein is an anti-HLA-G CAR comprising a humanized antigen-binding fragment that comprises:

(a) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 25; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 35;

(b) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 50; or

(c) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 60.

[0011] In some embodiments, the humanized antigen-binding fragment of an anti-HLA-G CAR is a single chain Fv (scFv). In some embodiments, the scFv comprises a sequence selected from SEQ ID NO: 63, SEQ ID NO: 65, or SEQ ID NO: 67.

[0012] In some embodiments, the CAR specifically binds β2M-associated HLA-G isoforms, preferably HLA-G1 and HLA-G5, preferably such CAR allows the discrimination of HLA-G isoforms, i.e. the CAR does not recognize or bind all of the seven HLA-G isoforms.

[0013] The disclosure particularly provides a humanized anti HLA-G CAR sequentially comprising from N to C terminus: (a) a peptide signal sequence, b) a humanized anti-HLA-G antibody or a humanized antigen binding fragment thereof, optionally c) a spacer domain, optionally d) a hinge domain, e) a transmembrane domain, f) an intracellular domain, and optionally g) a cleavable linker and optionally g) a truncated human CD19 domain. In some embodiments, anti HLA-G CAR comprises the sequence of SEQ ID NO: 68.

[0014] In one aspect, the spacer domain comprises (i) a human lgG4 hinge domain, (ii) a human lgG4 hinge domain and a CH3 human lgG4 domain or (iii) a mutated CH2 human lgG4 domain, a human lgG4 hinge domain and a CH3 human lgG4 hinge domain.

[0015] In another aspect, the signal peptide is selected from the group consisting of a CD8a signal peptide, a mouse Ig Kappa signal peptide, a human IgG4 signal peptide, an IL2 signal peptide, a human IgG2 signal peptide and a Gaussia luc signal peptide.

[0016] In some embodiments, the transmembrane domain is selected from CD28, CD3 and CD8 transmembrane domains, preferably the transmembrane domain is a CD28 transmembrane domain.

[0017] In some embodiments, the humanized anti HLA-G CAR of the disclosure comprises an intracellular domain that comprises a CD3 zeta signaling domain and at least one costimulatory domain(s) selected from CD28, 41BB, CD28, CD134, ICOS, OX40, CD149, DAP10, CD30, IL2-R, IL7r6, IL21-R, NKp30, NKp44, CD27, CD137 and DNAM-1 costimulatory domains, preferably the two costimulatory domains are 41BB and CD28 costimulatory domains.

[0018] In one aspect, the cleavable linker is selected from the group consisting of P2A, T2A, E2A, B2A and F2A.

[0019] In another aspect, the truncated human CD19 domain consists of the sequence set forth in SEQ ID No: 76.

[0020] Particularly, the CAR, the anti-HLA-G antibody or antigen binding fragment thereof, preferably a scFv, selectively binds to β2M-associated HLA-G isoforms but does not bind all HLA-G isoforms.

[0021] In another aspect, the CAR, the anti-HLA-G antibody or antigen binding fragment thereof, preferably a scFv, or the cell expressing CAR specifically bind the α1 domain of HLA-G.

[0022] Further provided herein is an anti-HLA-G antibody or an antigen binding fragment thereof, wherein the antibody or antigen-binding fragment comprises:

(a) (i) a VH comprising the sequence of SEQ ID NO: 25; and (ii) a VL comprising the sequence of SEQ ID NO: 35;

(b) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 50; or

(c) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 60.

[0023] Also provided herein is an anti- HLA-G antibody or an antigen-binding fragment thereof, wherein the antibody or antigen-binding fragment comprises:

(a) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 25; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 35;

(b) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 50; or

(c) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 60.

[0024] The disclosure also envisions a nucleic acid molecule encoding the CAR of the disclosure, to an expression vector, comprising the nucleic acid molecule and to a cell comprising the CAR of the disclosure or the nucleic acid molecule of the disclosure, or the expression vector of the disclosure, preferably wherein the cell is selected from a group consisting of a T cell, a CD4+ T cell, a CD8+ T cell, a B cell, a NK cell, a NKT cell, a monocyte and a dendritic cell, preferably the cell being a T cell, a B cell or a NK cell.

[0025] The disclosure also concerns a pharmaceutical composition comprising a nucleic acid molecule, an expression vector or a cell according to the disclosure and optionally a pharmaceutically acceptable carrier.

[0026] In some aspects, the cell of the disclosure or the pharmaceutical composition of the disclosure is for use in the treatment of cancer or for use in the treatment of a viral infection. The cell or pharmaceutical composition for such uses can be administered in combination with a CAR therapy that does not target HLA-G.

[0027] In one aspect, the pharmaceutical composition comprises a cell comprising a CAR specifically binding β2M-associated HLA-G, preferably to both HLA-G1 and HLA-G5.

[0028] Further provided herein is a method for treating cancer in a subject, comprising administering to the subject an effective amount of a pharmaceutical composition comprising a cell comprising a CAR as disclosed herein. In some embodiments, the cell is a T cell, a B cell, a NK cell, a NKT cell, a monocyte cell or a dendritic cell. In some embodiments, the cancer is HLA-G-expressing cancer. In some embodiments, the cancer is clear cell renal cell carcinoma, epithelial ovarian carcinoma, melanoma, kidney cancer, bladder cancer, breast cancer, ovarian cancer, uterine cancer, endometrial cancer, cervical cancer, renal cell cancer, colorectal cancer, gastric cancer, esophageal cancer, lung cancer, hepatocellular cancer, cholangiocarcinoma, neuroblastoma, cancer of the tongue, mouth and pharynx, bronchogenic cancer, cancer of the larynx, osteosarcoma, prostate cancer, testicular cancer, gastrointestinal stromal tumor, pancreatic cancer, kidney cancer, colon cancer, glioma, glioblastoma multiforme, medulloblastoma, thyroid cancer, adrenal carcinoma, acute myeloid leukemia, chronic lymphocytic leukemia, non-small cell lung cancer, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin lymphoma, B-cell lymphoma, monocytic lymphoma, marginal zone lymphoma, Burkitt's lymphoma, T cell lymphoma, B cell lymphoma, plasmacytoma, prohemocytic leukemia, acute non-lymphoblastic leukemia, acute lymphoblastic leukemia, erythroleukemia, myeloid leukemia or lymphoid leukemia.

[0029] Also provided herein is a cell comprising an anti-HLA-G CAR as disclosed herein or a composition as disclosed herein, for use as a medicament.

Brief Description of the Drawings

[0030] FIG. 1 is a schematic representation of the HLA-G CAR protein.

[0031] FIG. 2 shows humanness of LFTT-1 humanized variants, by homology (to parent and human germline) and by counting backmutations.

[0032] FIG. 3 shows humanness plotted against relative *IC50* score for all LFTT-1 humanized variants.

[0033] FIG. 4 shows specific cytotoxicity of anti-HLA-G CAR-T cells against multiple chemo resistant SKOV-3-HLA-G+ cell line.

[0034] FIG. 5A - FIG. 5C show *ex vivo* infiltration and activation of anti-HLA-G CAR-T cells in renal cell carcinoma tissues.

[0035] FIG. 6A - FIG. 6B show the effect of anti-HLA-G CAR-T cells on primary tumor control and elimination in an *in vivo* PDX model.

[0036] FIG. 7A-FIG. 7C show specific cytotoxicity of anti-HLA-G CAR-T cells derived from three donors against LCL-GFP-HLA-G cells.

[0037] FIG. 8A-FIG. 8C show the levels of IFN-γ secreted by anti-HLA-G CAR-T cells derived from three donors.

[0038] FIG. 9A-FIG. 9C. show specific cytotoxicity of anti-HLA-G CAR-T cells derived from three donors against LCL-HLA-G cells with the presence of increasing concentrations of soluble HLA-G.

Detailed Description

Overview

[0039] The present disclosure relates to a chimeric antigen receptor (CAR) comprising an extracellular domain, mostly constituted by a humanized antigen binding domain of an anti-HLA-G specific antibody, optionally a hinge domain comprising or consisting of (i) a human IgG4 hinge domain, (ii) a human IgG4 hinge domain and a CH3 human IgG4 domain or (iii) a mutated CH2 human IgG4 domain, a human IgG4 hinge domain and a CH3 human IgG4 hinge domain, a transmembrane domain, an intracellular domain that comprises one, two or three co-stimulatory structures, depending on the generation of the CAR design, optionally a cleavable linker and optionally a reporter.

[0040] Humanized anti-HLA-G CARs as disclosed herein exhibit reduced immunogenicity in human patients. In generating the humanized anti-HLA-G antibodies whose antigen binding domains are used in the CARs disclosed herein, it was necessary to consider both the humanness and relative *IC50* scores for each antibody as there is often a trade-off between binding efficiency and similarity to the human germline. Generally, more human-like designs lead to poorer binding efficiency as fewer murine residues are preserved in the designs, increasing the likelihood that the antibody will lose affinity for its target. More conservative designs generally lead to better binding efficiency. Provided herein are humanized anti-HLA-G CARs comprising antigen binding domains with lower relative *IC50* scores and a greater degree of humanness for retention of antibody affinity for the target and tolerance by the human immune system.

[0041] In some embodiments, the CAR according to the disclosure specifically binds to HLA-G isoforms associated β2M, preferably selected from HLA-G1 and HLA-G5.

[0042] Provided herein is a monoclonal antibody or a single chain variable fragment (scFv) molecule that specifically binds to both HLA-G1 and HLA-G5. It further relates to a monoclonal antibody or a single chain variable fragment (scFv) molecule that specifically binds to HLA-G isoforms associated β2M, preferably HLA-G1 and HLA-G5 isoforms.

[0043] The disclosure also concerns nucleic acid constructs or vectors containing the nucleic acid construct that can be transduced into a cell, preferably an immune cell such as a T cell, thereby creating a recombinant immune cell engineered to express the encoded CAR. Also provided are cells that are transduced to express the CAR of the disclosure, cell populations, and pharmaceutical compositions containing the cells expressing CAR.

[0044] In some embodiments, an immune effector cell is genetically modified to express an anti-HLA-G CAR. In some embodiments, genetically modified immune effector cells are administered to a subject with cancer cells expressing HLA-G including, but not limited to, solid tumors and hematological malignancies.

[0045] Provided herein are pharmaceutical compositions and formulations for administration, such as for adoptive cell therapy. Also provided are methods for preparing CAR expressing cells and administering the cells and compositions to subjects, e.g., patients.

[0046] Techniques for recombinant (i.e., engineered) DNA, peptide and oligonucleotide synthesis, immunoassays, tissue culture, transformation (e.g., electroporation, lipofection), enzymatic reactions, purification and related techniques and procedures may be generally performed as described in various general and more specific references in microbiology, molecular biology, biochemistry, molecular genetics, cell biology, virology and immunology as cited and discussed throughout the present specification. See, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 3d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Current Protocols in Molecular Biology (John Wiley and Sons, updated July 2008); Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience; Glover, DNA Cloning: A Practical Approach, vol. I & II (IRL Press, Oxford Univ. Press USA, 1985); Current Protocols in Immunology (Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober 2001 John Wiley & Sons, NY, NY); Real-Time PCR: Current Technology and Applications, Edited by Julie Logan, Kirstin Edwards and Nick Saunders, 2009, Caister Academic Press, Norfolk, UK; Anand, Techniques for the Analysis of Complex Genomes, (Academic Press, New York, 1992); Guthrie and Fink, Guide to Yeast

Genetics and Molecular Biology (Academic Press, New York, 1991); Oligonucleotide Synthesis (N. Gait, Ed., 1984); Nucleic Acid The Hybridization (B. Hames & S. Higgins, Eds., 1985); Transcription and Translation (B. Hames & S. Higgins, Eds., 1984); Animal Cell Culture (R. Freshney, Ed., 1986); Perbal, A Practical Guide to Molecular Cloning (1984); Next-Generation Genome Sequencing (Janitz, 2008 Wiley-VCH); PCR Protocols (Methods in Molecular Biology) (Park, Ed., 3rd Edition, 2010 Humana Press); Immobilized Cells And Enzymes (IRL Press, 1986); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Harlow and Lane, Antibodies, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998); Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir andCC Blackwell, eds., 1986); Roitt, Essential Immunology, 6th Edition, (Blackwell Scientific Publications, Oxford, 1988); Current Protocols in Immunology (Q. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach and W. Strober, eds., 1991); Annual Review of Immunology; as well as monographs in journals such as Advances in Immunology.

Definitions

[0047] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of particular embodiments, preferred embodiments of compositions, methods and materials are described herein. For the purposes of the present disclosure, the following terms are defined below. Additional definitions are set forth throughout this disclosure.

[0048] The articles "a," "an," and "the" are used herein to refer to one or to more than one (*i.e.*, to at least one, or to one or more) of the grammatical object of the article. By way of example, "an element" means one element or one or more elements.

[0049] The use of the alternative (*e.g.*, "or") should be understood to mean either one, both, or any combination thereof of the alternatives.

[0050] The term "and/or" should be understood to mean either one, or both of the alternatives.

[0051] Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device or the method being employed to determine the

value, or the variation that exists among the samples being measured. Unless otherwise stated or otherwise evident from the context, the term "about" means within 10% above or below the reported numerical value (except where such number would exceed 100% of a possible value or go below 0%). When used in conjunction with a range or series of values, the term "about" applies to the endpoints of the range or each of the values enumerated in the series, unless otherwise indicated. As used in this application, the terms "about" and "approximately" are used as equivalents.

[0052] A numerical range, e.g., 1 to 5, about 1 to 5, or about 1 to about 5, refers to each numerical value encompassed by the range. For example, in one non-limiting and merely illustrative embodiment, the range "1 to 5" is equivalent to the expression 1, 2, 3, 4, 5; or 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0; or 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5.0.

[0053] As used herein, the term "substantially" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that is 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher compared to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one embodiment, "substantially the same" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that produces an effect, e.g., a physiological effect, that is approximately the same as a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

[0054] Reference throughout this specification to "one embodiment," "an embodiment," "a particular embodiment," "a related embodiment," "a certain embodiment," "an additional embodiment," or "a further embodiment" or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments. It is also understood that the positive recitation of a feature in one embodiment, serves as a basis for excluding the feature in a particular embodiment.

HLA-G Antigen

[0055] "HLA-G" refers to human leukocyte antigen G. HLA-G is a non-classical HLA class I molecule that was first identified in choriocarcinoma cells. Unlike classic HLA class I molecules, HLA-G is characterized by a limited polymorphism and differs as well by its expression, structure and functions. Its expression is mainly restricted to the feto–maternal interface on the extravillous cytotrophoblast; to placenta, amnion; to a few healthy adult tissues such as thymus, cornea, bronchial epithelial cells, and pancreas; and to different types of cells such as mesenchymal stem cells, a few activated monocytes, and erythroid and endothelial precursors. The soluble HLA-G is also found in body fluids such as plasma, cerebrospinal fluid, malignant ascites, pleural effusions, and sperm. Although the HLA-G gene is not active in some tissues, its expression can be induced by certain molecules such as progesterone or anticancer drugs. Furthermore, this molecule can also be neo-expressed as well in pathological conditions such as cancer, multiple sclerosis, inflammatory diseases, and viral infections or after allograft. Soluble HLA-G (sHLA-G) can be detected in the serum/plasma of individuals.

[0056] HLA-G differs from classical HLA class I molecules by its low genetic diversity, a tissue-restricted expression, the existence of seven isoforms, and immuno-inhibitory functions. This molecule exerts an immuno-inhibitory function through direct binding to three inhibitory receptors: leukocyte immunoglobulin-like receptor B1 (LILRB1/ILT2/CD85j), LILRB2 (ILT4/CD85d) and KIR2DL4 (or CD158d). For LILRB receptors, the recognition site takes place through the α3 domain of HLA-G and it is unlikely affected by the peptide. LILRB1 is expressed by B cells, some T cells, some NK cells, and all monocytes/dendritic cells, whereas LILRB2 is myeloid specific and its expression is restricted to monocytes/dendritic cells. KIR2DL4 is a specific receptor for HLA-G, only expressed by the CD56bright subset of NK cells. LILRB1 and LILRB2 have been shown to bind a wide range of classic HLA molecules by the α3 domain and the β2M, for which HLA-G is the ligand of highest affinity, whereas for KIR2DL4, HLA-G is the sole known ligand. In addition, it has been demonstrated that LILRB1 and LILRB2 present higher affinity for HLA-G multimers than monomeric structures. It is important to bring up the difference between the way LILRB1 and LILRB2 bind to their ligands: LILRB1 shows higher affinity for HLA-G heavy chain associated to the β2M, whereas LILRB2 shows remarkably distinct MHCI-binding recognition by binding more the α3 domain than β2M, involving the aromatic amino acids Phe-195 and Tyr-197. This explains the β2M independent HLA-G binding of the latter receptor and its higher affinity for β2M free isoforms.

[0057] By linking these receptors, HLA-G acts as a down-regulator of the immune system for which some of the functions had been described: inhibition of the cytolytic function of uterine

and peripheral blood NK cells, the antigen-specific cytolytic function of cytotoxic T lymphocytes, the alloproliferative response of CD4+ T cells, the proliferation of T cells and peripheral blood NK cells, and the maturation and function of dendritic cells. Furthermore, HLA-G can induce the generation of suppressive cells. But, unlike classic HLA class I molecules, no stimulatory functions had been reported to date for HLA-G, neither responses directed against allogeneic HLA-G.

[0058] HLA-G can inhibit all the immune cell subsets; thus, it can block all the stages of the anti-tumor response. This molecule is expressed in many types of primary tumors, metastases and malignant effusions, and it can also be found on tumor cells and tumor-infiltrating cells. It was shown that HLA-G expression by tumor cell lines protects them from destruction by cytotoxic T lymphocytes and NK cells. Thus, the expression of HLA-G by malignant cells may prevent tumor immune elimination by inhibiting the activity of tumor infiltrating NK, cytotoxic T lymphocytes (CTL) and antigen presenting cells (APCs).

[0059] HLA-G expression is mainly controlled at the transcriptional level by a unique gene promoter and at the post-transcriptional level by alternative splicing, mRNA stability, translation and protein transport to the cell surface.

[0060] The primary transcript of HLA-G is alternatively spliced resulting in the expression of seven isoforms, where four are membrane-bound (HLA-G1, HLA-G2, HLA-G3 and HLA-G4) and three are soluble (HLA-G5, HLA-G6 and HLA-G7). HLA-G1 and HLA-G5 present the typical structure of a classical HLA class I molecule: a heavy chain constituted of three globular domains non-covalently bound to β2-microglobulin (β2M) and a peptide, while the other isoforms are shorter, lacking one or two domains of the heavy chain, and should not bind β2M.

[0061] HLA-G1 and HLA-G5 are considered the most abundant isoforms, probably because of the lack of antibodies diversity against other isoforms, particularly the lack of antibodies against β2M free isoforms.

[0062] HLA-G1 isoform is the complete isoform with α1, α2 and α3 domains associated with β2-microglobulin. The HLA-G2 isoform has no α2 domain, while HLA-G3 has no α2 and α3 domains, and HLA-G4 has no α3 domain. None of the isoforms HLA-G2, HLA-G3 and HLA-G4 binds β2M. The soluble HLA-G5 and HLA-G6 isoforms contain the same extra globular domains than HLA-G1 and HLA-G2, respectively. The HLA-G7 isoform has only the α1 domain linked to two amino acids encoded by intron 2. HLA-G5 isoform binds β2M while the isoforms HLA-G6 and HLA-G7 do not bind β2M.

[0063] In addition, HLA-G molecules can form dimers through the creation of disulfide bonds between two unique cysteine residues at positions 42 (Cys42-Cys42 bonds) and 147 (Cys42-

Cys147 bonds) of the HLA-G heavy chain. The dimerization has an oblique orientation that exposes the HLA-G receptor binding sites of the α3 domain upwards, making them more accessible to the receptors. Consequently, HLA-G dimers bind receptors with higher affinity and slower dissociation rates than monomers, and signal more efficiently than monomers as well.

[0064] Alternative names are HLA-G histocompatibility antigen class I or G or MHC-G. HLA-G is described in databases under the following accession numbers: Gene ID: 3135, UniGene Hs.512152. This protein is disclosed in UniProt under accession number: P17693. The GenBank entry of the sequence of the protein and mRNA are respectively NP_002118.1. and NM_002127.5.

When comparing HLA-G isoforms sequences, HLA-G1 is generally chosen as the canonical sequence, i.e. the sequence of DNA, RNA, or amino acids that reflects the most frequent nucleic acid or base or amino acid at each position, which is why database generally refer to this isoform sequence under the name "HLA-G". HLA-G2 to G7 differ from HLA-G1 by amino acid deletion(s) and/or substitution(s). HLA-G human isoforms are described under the Uniprot accession number P17693-1 for HLA-G1, P17693-2 for HLA-G2, P17693-3 for HLA-G3, P17693-4 for HLA-G4, P17693-5 for HLA-G5, P17693-6 for HLA-G6, P17693-7 for HLA-G7.

Antibodies against HLA-G isoforms

[0065] The present disclosure provides an antibody that specifically binds one to six, preferably two to five HLA-G isoform(s) among the seven HLA-G isoforms, but does not specifically bind or recognize all the HLA-G isoforms. For instance, the antibody can specifically bind HLA-G1, and HLA-G5 isoforms.

[0066] For example, the antibody or the antigen binding domain of the CAR according to the disclosure can recognize HLA-G1 and HLA-G5, if the epitope recognized by the antibody or antigen binding domain is on the β2M domain of HLA-G or on a domain which is specific of the HLA-G associated with the β2M domain.

[0067] In one aspect, the antibody can specifically bind HLA-G1 and HLA-G5 isoforms. Then, the antibody does not substantially bind the other HLA-G isoforms, especially HLA-G2, HLA-G3, HLA-G4, HLA-G6 and HLA-G7. More specifically, the antibody is specific of the HLA-G isoforms associated with β2M. In this context, the antibody does not substantially bind HLA-G1 and HLA-G5 isoforms devoid of β2M.

[0068] It is provided herein a humanized antibody LFTT-1. In particular, humanized LFTT-1 is a humanized scFv antibody. LFTT-1 is described in WO2020043899, which is incorporated herein by reference in its entirety. The CDRs of the humanized antibody LFTT-1 have the following sequences, according to Kabat:

(a) Heavy chain CDR1 of SEQ ID NO: 11;

(b) Heavy chain CDR2 of SEQ ID NO: 13,

(c) Heavy chain CDR3 of SEQ ID NO: 15,

(d) Light chain CDR1 of SEQ ID NO: 37,

(e) Light chain CDR2 of SEQ ID NO: 39, and

(f) Light chain CDR3 of SEQ ID NO: 41;

wherein each CDR may optionally comprise 1, 2, 3 or 4 amino acid substitutions, deletions or insertions.

[0069] Accordingly, the present disclosure relates to anti-HLA-G antibody or antigen binding fragment thereof that is an anti-HLA-G scFv comprising:

(a) (i) a VH comprising the sequence of SEQ ID NO: 25; and (ii) a VL comprising the sequence of SEQ ID NO: 35;

(b) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 50; or

(c) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 60.

[0070] Said antibody can be a chimeric, human or humanized. Said antibody can be an antibody fragment selected from Fab, Fab', Fab'-SH, F(ab') 2, Fv, a diabody, or a single-chain antibody fragment, comprising multiple different antibody fragments. Said antibody can be conjugated or covalently bound to a toxic agent or to a detectable label.

[0071] In some embodiments, the antibody or fragment thereof binds one or several but not all HLA-G isoform(s) with high affinities of at least about 10^7 M^{-1}, and preferably at least about 10^8 M^{-1}, 10^9 M^{-1}, 10^{10} M^{-1}.

[0072] In some embodiments, the antibody or fragment thereof does not bind or recognize the α1 domain of HLA-G isoforms. This means that such antibody or fragment thereof can bind or recognize HLA-G isoforms lacking of α1 domain, for example HLA-G isoforms such as

described in Tronik-Le Roux et al., Molecular Oncology 11 (2017) 1561-1578, that contain the α2 and α3 domains or only the α3 domain. For example, such antibody or fragment thereof can bind the α2, α3 or β2M domain.

[0073] The sequence of the antibody or antibody fragment according to the disclosure may be used in a method to prepare a CAR or to prepare a pharmaceutical composition. Alternatively, the antibody or antibody fragment according to the disclosure may be used to detect HLA-G isoform(s) in diagnosis tests such as immunoassays.

[0074] Antibodies or antibody fragments can be identified, for example, by immunoassays such as radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs) and Surface Plasmon Resonance (SPR) assays or other techniques known to those of skill in the art.

[0075] Preferably, antibodies (including antibody fragments thereof) that specifically bind to one or several HLA-G isoform(s) do not significantly cross-react with other antigens (i.e., is not detectable in routine immunological assays). An antibody binds specifically to an antigen when it binds to the antigen with higher affinity than to any cross-reactive antigen as determined using experimental techniques, such as Western blot (WB), radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs), particularly competitive ELISA.

Chimeric Antigen Receptors

[0076] In various embodiments, genetically engineered receptors that redirect cytotoxicity of immune effector cells toward cancer cells expressing human leukocyte antigen G (HLA-G) are provided herein. These genetically engineered receptors are referred to herein as chimeric antigen receptors (CARs). CARs are artificially constructed hybrid proteins or polypeptides that combine binding specificity for a desired antigen (*e.g.*, HLA-G) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-HLA-G cellular immune activity and activate the T cell upon interaction with the target antigen (*e.g.*, HLA-G).

[0077] Prototypic single chain CARs were first described in a study by Eshhar and colleagues in 1993 in which specific activation and targeting of T cells was mediated through molecules consisting of a target-antigen-specific antibody domain and the γ- or ζ-signaling subunits of the Fc epsilon receptor or T-cell receptor CD3 complex, respectively (Eshhar et al., 1993, Proc Natl Acad Sci USA, 90, 720-4). In more recent versions, the binding domain of a CAR typically consists of an antigen-binding domain of a single-chain antibody (scFv) or antibody-binding

fragment (Fab) selected from a library and comprising the light and heavy chain variable fragments of a monoclonal antibody (Mabs) joined by a flexible linker. The scFv retains the same specificity and a similar affinity as the full antibody from which it was derived and is able to specifically bind to the target of interest. CARs thus combine antigen-specificity and T cell activating properties in a single fusion molecule. Indeed, the scFv is linked to an intracellular signaling module that includes CD3ζ to induce T cell activation upon antigen binding. The modular structure has been extended from first-generation CARs with only a CD3ζ signaling domain to second and third generation CARs that link the signaling endodomains such as CD28, 4-1BB, or OX40 to CD3ζ, in an attempt to mimic co-stimulation. Generally, a spacer or hinge domain serves as a linker between the endodomains and the scFv. The incorporation of such hinge domain improves flexibility, spatial organization and/or proximity but also the expansion of CAR cells (Qin et al, Journal of Hematology & Oncology. 2017; 10:68) or tumor localization (Watanabe et al. Oncoimmunology. 2016; 5(12): e1253656).

[0078] In some embodiments, CARs disclosed herein comprise an extracellular domain (comprising a binding domain or antigen-specific binding domain) that binds to HLA-G, a transmembrane domain, and an intracellular domain. In some embodiments, a CAR comprises, in amino-terminal to carboxyl-terminal order (a) an extracellular domain that binds to HLA-G, (b) a transmembrane domain, and (c) an intracellular domain. Engagement of the anti-HLA-G antigen binding domain of the CAR with HLA-G on the surface of a target cell delivers an activation stimulus to the CAR-expressing cell. In some embodiments, engagement of the anti-HLA-G antigen binding domain of the CAR with HLA-G on the surface of a target cell results in clustering of the CAR and the subsequent activation of the CAR-expressing cell.

[0079] The main characteristic of CARs is their ability to exploit the cell specific targeting abilities of monoclonal antibodies, soluble ligands or cell specific co-receptors by redirecting immune effector cell specificity, thereby triggering proliferation, cytokine production, phagocytosis, and/or production of molecules that can mediate cell death of the target antigen expressing cell in a major histocompatibility (MHC) independent manner. The non-MHC-restricted antigen recognition gives T cells expressing CARs the ability to recognize antigen independent of antigen processing, thus bypassing a major mechanism of tumor escape. Moreover, when expressed in T-cells, CARs advantageously do not dimerize with endogenous T cell receptor (TCR) alpha and beta chains.

Binding Domain

[0080] In some embodiments, CARs comprise an extracellular domain that comprises an antigen-binding domain that specifically binds to HLA-G (*e.g.*, human HLA-G). For example, the HLA-G may be a human HLA-G polypeptide expressed on a target cell, *e.g.*, a cancer cell. In some embodiments, a CAR antigen-binding domain is an anti-HLA-G antibody or antigen-binding fragment thereof. As used herein, the terms, "binding domain," "antigen-binding domain," "extracellular domain," "extracellular binding domain," "antigen-specific binding domain," and "extracellular antigen specific binding domain," are used interchangeably and provide a CAR with the ability to specifically bind to the target antigen of interest, *e.g.*, HLA-G. The binding domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source.

[0081] The terms "specific binding affinity" or "specifically binds" or "specifically bound" or "specific binding" or "specifically targets" as used herein, describe binding of an anti-HLA-G antibody or antigen binding fragment thereof (or a CAR comprising the same) to HLA-G at greater binding affinity than background binding. A binding domain (or a CAR comprising a binding domain or a fusion protein containing a binding domain) "specifically binds" to an HLA-G polypeptide if it binds to or associates with HLA-G with an affinity or K_a (*i.e.*, an equilibrium association constant of a particular binding interaction with units of 1/M) of, for example, greater than or equal to about 10^5 M^{-1}. In certain embodiments, a binding domain (or a fusion protein thereof) binds to a target with a K_a greater than or equal to about 10^6 M^{-1}, 10^7 M^{-1}, 10^8 M^{-1}, 10^9 M^{-1}, 10^{10} M^{-1}, 10^{11} M^{-1}, 10^{12} M^{-1}, or 10^{13} M^{-1}. "High affinity" binding domains (or single chain fusion proteins thereof) refers to those binding domains with a K_a of at least 10^7 M^{-1}, at least 10^8 M^{-1}, at least 10^9 M^{-1}, at least 10^{10} M^{-1}, at least 10^{11} M^{-1}, at least 10^{12} M^{-1}, at least 10^{13} M^{-1}, or greater.

[0082] Alternatively, affinity may be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (*e.g.*, 10^{-5} M to 10^{-13} M, or less). Affinities of binding domain polypeptides and CAR proteins according to the present disclosure can be readily determined using conventional techniques, *e.g.*, by competitive ELISA (enzyme-linked immunosorbent assay), or by binding association, or displacement assays using labeled ligands, or using a surface-plasmon resonance device such as the BIACORE® T100, which is available from Biacore, Inc., Piscataway, NJ, or optical biosensor technology such as the EPIC system or EnSpire that are available from Corning and Perkin Elmer respectively (*see also, e.g.*,

Scatchard *et al.* (1949) Ann. N.Y. Acad. Sci. 51:660; and U.S. Patent Nos. 5,283,173; 5,468,614, or the equivalent).

[0083] In some embodiments, the extracellular binding domain of a CAR comprises an antibody or antigen binding fragment thereof. An "antibody" refers to a binding agent that is a polypeptide comprising at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and binds an epitope of an antigen, such as a peptide, lipid, polysaccharide, or nucleic acid containing an antigenic determinant, such as those recognized by an immune cell. The term also includes genetically engineered forms such as chimeric antibodies (for example, humanized murine antibodies), heteroconjugate antibodies (such as, bispecific antibodies) and antigen binding fragments thereof. See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, IL); Kuby, J., Immunology, 3rd Ed., W. H. Freeman & Co., New York, 1997.

[0084] An "antigen (Ag)" refers to a compound, composition, or substance that can stimulate the production of antibodies or a T cell response in an animal, including compositions (such as one that includes a cancer-specific protein) that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous antigens, such as the disclosed antigens. In some embodiments, the target antigen is an epitope of an HLA-G polypeptide.

[0085] An "epitope" or "antigenic determinant" refers to the region of an antigen to which a binding agent binds. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5, about 9, or about 8-10 amino acids in a unique spatial conformation

[0086] A "monoclonal antibody" is an antibody produced by a single clone of B lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. Monoclonal antibodies include humanized monoclonal antibodies.

[0087] A "chimeric antibody" has framework residues from one species, such as human, and CDRs (which generally confer antigen binding) from another species, such as a mouse. In some

embodiments, a CAR comprises antigen-specific binding domain that is a chimeric antibody or antigen binding fragment thereof.

[0088] In some embodiments, the antibody is a human antibody (such as a human monoclonal antibody) or fragment thereof that specifically binds to a human HLA-G polypeptide. Human antibodies can be constructed by combining Fv clone variable domain sequence(s) selected from human-derived phage display or yeast display libraries with known human constant domain sequences(s) as described above. Alternatively, human monoclonal antibodies may be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner *et al.*, *J. Immunol.*, 147: 86 (1991). In addition, transgenic animals (*e.g.*, mice) can be used to produce a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. See, *e.g.*, Jakobovits *et al.*, *PNAS USA*, 90: 2551 (1993); Jakobovits *et al.*, *Nature*, 362: 255 (1993); Bruggermann *et al.*, *Year in Immunol.*, 7: 33 (1993). Gene shuffling can also be used to derive human antibodies from non-human, *e.g.*, rodent antibodies, where the human antibody has similar affinities and specificities to the starting non-human antibody. (*See* WO 93/06213). Unlike traditional humanization of non-human antibodies by CDR grafting, this technique provides completely human antibodies, which have no FR or CDR residues of non-human origin.

[0089] In one embodiment, a CAR comprises a "humanized" antibody. A humanized antibody is an immunoglobulin including a human framework region and one or more CDRs from a non-human (for example a mouse, rat, or synthetic) immunoglobulin. The non-human immunoglobulin providing the CDRs is termed a "donor," and the human immunoglobulin providing the framework is termed an "acceptor." In one embodiment, all the CDRs are from the donor immunoglobulin in a humanized immunoglobulin. Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, *i.e.*, at least about 85-90%, such as about 95% or more identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. Humanized or other monoclonal antibodies can have additional conservative amino acid substitutions, which have substantially no effect on antigen binding or other immunoglobulin functions. Humanized

antibodies can be constructed by means of genetic engineering (see for example, U.S. Patent No. 5,585,089).

[0090] In some embodiments, a CAR comprises one or more humanized framework regions (FRs). In some embodiments, an anti-HLA-G CAR comprises a heavy chain variable region comprising one, two, three, or four heavy chain FRs that have been humanized. In some embodiments, an anti-HLA-G CAR comprises a heavy chain variable region comprising, in order from N-terminus to C-terminus: a humanized heavy chain FR1 as set forth in SEQ ID NO: 10 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 10; a CDR1 as set forth in SEQ ID NO: 11; a humanized heavy chain FR2 as set forth in one of SEQ ID NOs: 12 and 26 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 12 or 26; a CDR2 as set forth in SEQ ID NO: 13; a humanized heavy chain FR3 as set forth in SEQ ID NO: 14 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 14; a CDR3 as set forth in SEQ ID NO: 15; and a humanized heavy chain FR4 as set forth in SEQ ID NO: 16 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 16. In some embodiments, an anti-HLA-G CAR comprises a light chain variable region comprising one, two, three, or four light chain FRs that have been humanized. In some embodiments, an anti-HLA-G CAR comprises a light chain variable region comprising, in order from N-terminus to C-terminus: a humanized light chain FR1 as set forth in SEQ ID NO: 36 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 36; a CDR1 as set forth in SEQ ID NO: 37; a humanized light chain FR2 as set forth in SEQ ID NO: 38 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 38; a CDR2 as set forth in SEQ ID NO: 39; a humanized light chain FR3 as set forth in one of SEQ ID NOs: 40 and 51 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 40 or 51; a CDR3 as set forth in SEQ ID NO: 41; and a humanized light chain FR4 as set forth in SEQ ID NO: 42 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 42.

[0091] Antigen binding fragments include Camel Ig, Ig NAR, Fab fragments, Fab' fragments, $F(ab')_2$ fragments, bispecific Fab dimers (Fab2), trispecific Fab trimers (Fab3), Fv, single chain Fv proteins ("scFv"), bis-scFv, $(scFv)_2$, minibodies, diabodies, triabodies, tetrabodies, disulfide stabilized Fv proteins ("dsFv"), and single-domain antibody (sdAb, Nanobody) and portions of full length antibodies responsible for antigen binding. An "isolated antibody or antigen binding fragment thereof" is one which has been identified and separated and/or recovered from a component of its natural environment.

[0092] As would be understood by the skilled person and as described elsewhere herein, a complete antibody comprises two heavy chains and two light chains. Each heavy chain consists

of a variable region and a first, second, and third constant region, while each light chain consists of a variable region and a constant region. Mammalian heavy chains are classified as α, δ, ε, γ, and μ. Mammalian light chains are classified as λ or κ. Immunoglobulins comprising the α, δ, ε, γ, and μ heavy chains are classified as immunoglobulin (Ig)A, IgD, IgE, IgG, and IgM. The complete antibody forms a "Y" shape. The stem of the Y consists of the second and third constant regions (and for IgE and IgM, the fourth constant region) of two heavy chains bound together and disulfide bonds (inter-chain) are formed in the hinge. Heavy chains γ, α and δ have a constant region composed of three tandem (in a line) Ig domains, and a hinge region for added flexibility; heavy chains μ and ε have a constant region composed of four immunoglobulin domains. The second and third constant regions are referred to as "CH2 domain" and "CH3 domain", respectively. Each arm of the Y includes the variable region and first constant region of a single heavy chain bound to the variable and constant regions of a single light chain. The variable regions of the light and heavy chains are responsible for antigen binding.

[0093] Light and heavy chain variable regions contain a "framework" region interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs." The CDRs can be defined or identified by conventional methods, such as by sequence according to Kabat *et al.* (Wu, TT and Kabat, E. A., *J Exp Med.* 132(2):211-50, (1970); Borden, P. and Kabat E. A., *PNAS*, 84: 2440-2443 (1987); (see, Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, U.S. Department of Health and Human Services, 1991, which is hereby incorporated by reference), or by structure according to Chothia *et al* (Chothia, C. and Lesk, A.M., *J Mol. Biol.*, 196(4): 901-917 (1987), Chothia, C. *et al*, *Nature*, 342: 877 - 883 (1989)).

[0094] The sequences of the framework regions of different light or heavy chains are relatively conserved within a species, such as humans. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three-dimensional space. The CDRs are primarily responsible for binding to an epitope of an antigen. The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are also typically identified by the chain in which the particular CDR is located. Thus, the CDRs located in the variable domain of the heavy chain of the antibody are referred to as HCDR1, HCDR2, and HCDR3, whereas the CDRs located in the variable domain of the light chain of the antibody are referred to as LCDR1, LCDR2, and LCDR3. Antibodies with different specificities (*i.e.*,

different combining sites for different antigens) have different CDRs. Although it is the CDRs that vary from antibody to antibody, only a limited number of amino acid positions within the CDRs are directly involved in antigen binding. These positions within the CDRs are called specificity determining residues (SDRs). Illustrative examples of light chain CDRs that are suitable for constructing anti-HLA-G CARs contemplated in some embodiments include, but are not limited to, the CDR sequences set forth in SEQ ID NOs: 37, 39, and 41. Illustrative examples of heavy chain CDRs that are suitable for constructing anti-HLA-G CARs contemplated in some embodiments include, but are not limited to the CDR sequences set forth in SEQ ID NOs: 11, 13, and 15.

[0095] References to "V_H" or "VH" refer to the variable region of an immunoglobulin heavy chain, including that of an antibody, Fv, scFv, dsFv, Fab, or other antibody fragment as contemplated herein. Illustrative examples of heavy chain variable regions that are suitable for constructing anti-HLA-G CARs contemplated in some embodiments include, but are not limited to, the heavy chain variable region sequences set forth in SEQ ID NOs: 9 and 25.

[0096] References to "V_L" or "VL" refer to the variable region of an immunoglobulin light chain, including that of an antibody, Fv, scFv, dsFv, Fab, or other antibody fragment as contemplated herein. Illustrative examples of light chain variable regions that are suitable for constructing anti-HLA-G CARs contemplated in some embodiments include, but are not limited to, the light chain variable region sequences set forth in SEQ ID NOs:35, 50, and 60.

[0097] In some embodiments, an anti-HLA-G antibody or antigen binding fragment thereof, includes but is not limited to a Camel Ig (a camelid antibody (VHH)), Fab fragments, Fab' fragments, F(ab)'2 fragments, F(ab)'3 fragments, Fv, single chain Fv antibody ("scFv"), bis-scFv, (scFv)2, minibody, diabody, triabody, tetrabody, disulfide stabilized Fv protein ("dsFv"), single-domain antibody (sdAb, Nanobody) and a shark antibody domain.

[0098] "Camel Ig" or "camelid VHH" as used herein refers to the smallest known antigen-binding unit of a heavy chain antibody (Koch-Nolte, *et al*, FASEB J., 21: 3490-3498 (2007)). A "heavy chain antibody" or a "camelid antibody" refers to an antibody that contains two VH domains and no light chains (Riechmann L. *et al*, J. Immunol. Methods 231:25–38 (1999); WO94/04678; WO94/25591; U.S. Patent No. 6,005,079). In some embodiments, an antigen-binding domain is a camelid nanobody.

[0099] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose

name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

[00100] "Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three hypervariable regions (HVRs) of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six HVRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[00101] The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known. Bispecific Fab dimers (Fab2) have two Fab' fragments, each binding a different antigen. Trispecific Fab trimers (Fab3) have three Fab' fragments, each binding a different antigen.

[00102] The term "diabodies" refers to antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO 1993/01161; Hudson *et al.*, *Nat. Med.* 9:129-134 (2003); and Hollinger *et al.*, *PNAS USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson *et al.*, *Nat. Med.* 9:129-134 (2003).

[00103] "Single domain antibody" or "sdAb" or "nanobody" refers to an antibody fragment that consists of the variable region of an antibody heavy chain (VH domain) or the variable region of an antibody light chain (VL domain) (Holt, L., *et al*, T*rends in Biotechnology*, 21(11): 484-490).

[00104] "Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain and in either orientation (*e.g.*, VL-VH or VH-VL). Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see, *e.g.*, Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York, 1994), pp. 269-315.

[00105] In some embodiments, the anti-HLA-G antigen binding fragment is a scFv. In some embodiments, the scFv is a murine, human or humanized scFv. Single chain antibodies may be cloned form the V region genes of a hybridoma specific for a desired target. The production of such hybridomas has become routine. A technique which can be used for cloning the variable region heavy chain (V_H) and variable region light chain (V_L) has been described, for example, in Orlandi *et al., PNAS,* 1989; 86: 3833-3837.

[00106] In some embodiments, a CAR antigen-binding domain comprises a heavy chain variable (VH) region comprising the HCDR1, HCDR2 and HCDR3 and a light chain variable (VL) region comprising the LCDR1, LCDR2 and LCDR3 of an anti-HLA-G antibody. In some embodiments, an anti-HLA-G antibody or antigen binding fragment thereof comprises a variable heavy chain sequence comprising a HCDR1 sequence set forth in SEQ ID NO: 11, a HCDR2 sequence set forth in SEQ ID NO: 13, and a HCDR3 sequence set forth in SEQ ID NO: 15. In some embodiments, an anti-HLA-G antibody or antigen binding fragment thereof comprises a variable light chain sequence comprising a LCDR1 sequence set forth in SEQ ID NO: 37, a LCDR2 sequence set forth in SEQ ID NO: 39, and a LCDR3 sequence set forth in SEQ ID NO: 41. In some embodiments, the anti-HLA-G antibody or antigen binding fragment thereof comprises a variable heavy chain sequence as set forth in any one of SEQ ID NOs: 9 and 25 and/or a variable light chain sequence as set forth in any one of SEQ ID NOs: 35, 50 and 60.

[00107] In some embodiments, HLA-G-binding domains may comprise an antibody mimetic. The term "antibody mimetic" can describe an organic compound that specifically binds a target

sequence and has a structure distinct from a naturally-occurring antibody. Antibody mimetics may comprise a protein, a nucleic acid, or a small molecule. The target sequence to which an antibody mimetic specifically binds may be HLA-G. Antibody mimetics may provide superior properties over antibodies including, but not limited to, superior solubility, tissue penetration, stability towards heat and enzymes (e.g., resistance to enzymatic degradation), and lower production costs. Exemplary antibody mimetics include, but are not limited to, an affibody, an affilin, an affimer, an affitin, an alphabody, an anticalin, and an avimer (also known as avidity multimer), a DARpin® (Designed Ankyrin Repeat Protein), a Fynomer, a Kunitz domain peptide, and a monobody.

Linkers

[00108] In certain embodiments, anti-HLA-G CARs comprise linker residues between the various domains, *e.g.*, added for appropriate spacing and conformation of the molecule. In some embodiments, CARs comprise one, two, three, four, or five or more linkers. In particular some, the length of a linker is about 1 to about 25 amino acids, about 5 to about 20 amino acids, or about 10 to about 20 amino acids, or any intervening length of amino acids. In some embodiments, the linker is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more amino acids long.

[00109] Illustrative examples of linkers include glycine polymers $(G)_n$; glycine-serine polymers $(G_{1-5}S_{1-5})_n$, where n is an integer of at least one, two, three, four, or five; glycine-alanine polymers; alanine-serine polymers; and other flexible linkers known in the art. Glycine and glycine-serine polymers are relatively unstructured, and therefore may be able to serve as a neutral tether between domains of fusion proteins such as the CARs described herein. Glycine accesses significantly more phi-psi space than even alanine, and is much less restricted than residues with longer side chains (*see* Scheraga, *Rev. Computational Chem.* 11173-142 (1992)). The ordinarily skilled artisan will recognize that design of a CAR in some embodiments can include linkers that are all or partially flexible, such that the linker can include a flexible linker as well as one or more portions that confer less flexible structure to provide for a desired CAR structure. In some embodiments, a linker that links the heavy chain variable region and light chain variable region comprises the sequence of SEQ ID NO: 70.

[00110] In some embodiments, the CAR according to the disclosure comprises a cleavable linker. The cleavable linker may be a peptide, a polypeptide or a part of a polypeptide, which is cleaved after the generation of the protein or polypeptide, particularly, after the translation of the CAR according to the disclosure.

[00111] Particularly, the cleavable linker is a self-cleavable, self-cleaving, self-cleavage peptide or linker, these terms being used interchangeably herein.

[00112] In one embodiment, the cleavable linker comprises a 2A peptide. "2A" or "2A-like" sequences are part of a large family of peptides that can cause peptide bond-skipping. Particularly, the mechanism of 2A-mediated "self-cleavage" was recently discovered to be ribosome skipping the formation of a glycyl-prolyl peptide bond at the C-terminus of the 2A peptide. The 2A-peptide-mediated cleavage commences after the translation. Successful skipping and recommencement of translation results in two "cleaved" proteins: the protein upstream of the 2A is attached to the complete 2A peptide except for the C-terminal proline, and the protein downstream of the 2A is attached to one proline at the N-terminus. Successful skipping but ribosome fall-off and discontinued translation results in only the protein upstream of 2A. Several 2A peptides have been identified in picornaviruses, insect viruses and type C rotaviruses.

[00113] Examples of cleavable linker according to the disclosure include, but are not limited to, porcine teschovirus-1 2A (P2A), FMDV 2A (F2A); equine rhinitis A virus (ERAV) 2A (E2A); and Thosea asigna virus 2A (T2A), cytoplasmic polyhedrosis virus 2A (BmCPV2A) and flacherie Virus 2A (BmIFV2A), or a combination thereof, for example such as described in Kim et al. (2011) PLoS ONE 6(4): e18556 and in Liu et al (2017) Sci Rep. 2017; 7: 2193.

[00114] Preferably, the cleavable linker is P2A which comprises or consists of the sequence set forth in SEQ ID NO: 75 or a sequence having at least 80, 85, 90 or 95% of identity therewith.

[00115] In one embodiment, the N-terminus of the cleavable linker is operably linked to the C-terminus of the CAR endodomain and/or the C-terminus of the cleavable linker is operably linked to the N-terminus of a reporter.

Signal peptide

[00116] In some embodiments, the extracellular domain of an anti-HLA-G CAR comprises a signal peptide. In some embodiments, the signal peptide comprises a sequence encoding a human CD2, CD3δ, CD3ε, CD3γ, CD3ζ, CD4, CD8α, CD19, CD28, CD37, CD45, 4-1BB,

GM-CSFR, IL-2, CD33, Human IgKVIII, Human IgG2 H, Chymotrypsinogen, trypsinogen-2, HSA, Insulin or tPA signal peptide. In some embodiments, a signal peptide of an anti-HLA-G CAR comprises the sequence of SEQ ID NO: 69.

Spacer domain

[00117] In some embodiments, the extracellular domain of an anti-HLA-G CAR comprises one or more "spacer domains," which refers to the region that moves the antigen binding domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation (Patel *et al.*, *Gene Therapy*, 1999; 6: 412-419). In some embodiments, a CAR comprises a spacer domain between an antigen-binding domain and a transmembrane (TM) domain. The spacer domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. In certain embodiments, a spacer domain is a portion of an immunoglobulin, including, but not limited to, one or more heavy chain constant regions, *e.g.*, CH2 and CH3. The spacer domain can include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region. In one embodiment, the spacer domain comprises the CH2 and CH3 of IgG1, IgG4, or IgD.

Hinge domain

[00118] In some embodiments, the extracellular domain of an anti-HLA-G CAR comprises one or more "hinge domains," which play a role in positioning the antigen binding domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation. An anti-HLA-G CAR generally comprises one or more hinge domains between the antigen-binding domain and the transmembrane (TM) domain. The hinge domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. The hinge domain can include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

[00119] In some embodiments, the hinge domain may be derived from or include at least a portion of an immunoglobulin Fc region, for example, an IgG1 Fc region, an IgG2 Fc region, an IgG3 Fc region, an IgG4 Fc region, an IgE Fc region, an IgM Fc region, or an IgA Fc region. In certain embodiments, the hinge domain includes at least a portion of an IgG1, an IgG2, an IgG3, an IgG4, an IgE, an IgM, or an IgA immunoglobulin Fc region that falls within its CH2 and CH3 domains. In some embodiments, the spacer domain may also include at least a portion of a corresponding immunoglobulin hinge region. In some embodiments, the hinge is derived from or includes at least a portion of a modified immunoglobulin Fc region, for example, a

modified IgG1 Fc region, a modified IgG2 Fc region, a modified IgG3 Fc region, a modified IgG4 Fc region, a modified IgE Fc region, a modified IgM Fc region, or a modified IgA Fc region. The modified immunoglobulin Fc region may have one or more mutations (e.g., point mutations, insertions, deletions, duplications) resulting in one or more amino acid substitutions, modifications, or deletions that cause impaired binding of the spacer domain to an Fc receptor (FcR). In some aspects, the modified immunoglobulin Fc region may be designed with one or more mutations which result in one or more amino acid substitutions, modifications, or deletions that cause impaired binding of the spacer domain to one or more FcR including, but not limited to, FcγRI, FcγR2A, FcγR2B1, FcγR2B2, FcγR3A, FcγR3B, FcεRI, FcεR2, FcαRI, Fcα/μK, or FcRn.

[00120] Exemplary hinges include, but are not limited to, a CD8α hinge, a CD28 hinge, IgG1/IgG4 (hinge-Fc part) sequences (in single studies, CD4, CD7, and IgD) IgG4 hinge alone, IgG4 hinge linked to CH2 and CH3 domains, or IgG4 hinge linked to the CH3 domain, those described in Hudecek et al. (2013) Clin. Cancer Res., 19:3153, international patent application publication number WO2014031687, U.S. Pat. No. 8,822,647 or published app. No. US2014/0271635. As hinge domain, the disclosure relates to all or a part of residues 118 to 178 of CD8a (GenBank Accession No. NP_001759.3), residues 135 to 195 of CD8 (GenBank Accession No. AAA35664), residues 315 to 396 of CD4 (GenBank Accession No. NP_000607.1), or residues 137 to 152 of CD28 (GenBank Accession No. NP_006130.1) can be used. Also, as the spacer domain, a part of a constant region of an antibody H chain or L chain (CHI region or CL region) can be used. Further, the spacer domain may be an artificially synthesized sequence. Particularly, the CAR according to the disclosure comprises a hinge selected from CD8a, CD28, and IgG1/IgG4 (hinge-Fc part) sequences (in single studies, CD4, CD7, and IgD). This choice is based on the fact that these sequences are relatively neutral, flexible, and have been well-characterized structurally.

[00121] Preferably, the hinge domain comprises or consists of (i) CD28 hinge, (ii) CD8 alpha hinge, (iii) a human lgG4 hinge domain, (iv) a human lgG4 hinge domain and a CH3 human lgG4 domain or (v) a mutated CH2 human lgG4 domain, a human lgG4 hinge domain and a CH3 human lgG4 hinge domain. In one embodiment, the hinge domain comprises the sequence of SEQ ID NO: 71, or a sequence having at least 80, 85, 90 or 95% identity therewith.

[00122] In one embodiment, the hinge domain comprises or consists of (i) a human lgG4 hinge domain, (ii) a human lgG4 hinge domain and a CH3 human lgG4 domain or (iii) a mutated CH2 human lgG4 domain, a human lgG4 hinge domain and a CH3 human lgG4 hinge domain.

Particularly, the hinge domain sequentially comprises or consists from the N terminus to the C terminus of (i) a human lgG4 hinge domain and a CH3 human lgG4 domain or (ii) a mutated CH2 human lgG4 domain, a human lgG4 hinge domain and a CH3 human lgG4 hinge domain.

Transmembrane Domain

[00123] The "transmembrane (TM) domain" or "transmembrane (TM) region" is the portion of an anti-HLA-G CAR that fuses the extracellular binding portion and intracellular signaling domain and anchors the CAR to the plasma membrane of the immune effector cell. The TM domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. The TM domain may be derived from (*i.e.*, comprise at least the transmembrane region(s) of) the alpha or beta chain of the T-cell receptor, CD2, CD3δ, CD3ε, CD3γ, CD3ζ, CD4, CD5, CD8α, CD9, CD16, CD19, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD134, CD137, CD152, CD154, 4-1BB, GM-CSFR, PD1, or FcRIγ and less frequently CD7, OX40, and MHC (H2-Kb), the choice depending on the neighboring spacer and intracellular sequences. Persons of skill are aware of numerous transmembrane regions and the structural elements (such as lipophilic amino acid regions) that produce transmembrane domains in numerous membrane proteins and therefore can substitute any convenient sequence.

[00124] In an embodiment of the disclosure, the transmembrane domain comprises a CD8α transmembrane domain or a CD28 transmembrane domain. In some embodiments, the CD8 and CD28 are derived from the human CD8α or CD28 sequences. The CD8α or CD28 may comprise less than the whole CD8α or CD28, respectively. In this regard, in some embodiments, the CAR comprises a CD28 transmembrane domain comprising, consisting of, or consisting essentially of SEQ ID NO: 72, or a sequence having at least 80, 85, 90 or 95% of identity therewith.

[00125] Alternatively, the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. A transmembrane domain of the disclosure is thermodynamically stable in a membrane. It may be a single alpha helix, a transmembrane beta barrel, a beta-helix of gramicidin A, or any other structure.

[00126] Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the intracellular signaling domain(s) of the CAR. A glycine-serine doublet may provide a suitable linker.

Intracellular Domain

[00127] In some embodiments, anti-HLA-G CARs comprise an intracellular domain. An "intracellular domain," refers to the part of a CAR that participates in transducing the message of effective anti-HLA-G CAR binding to a human HLA-G polypeptide into the interior of the immune effector cell to elicit effector cell function, *e.g.*, activation, cytokine production, proliferation and cytotoxic activity, including the release of cytotoxic factors to the CAR-bound target cell, or other cellular responses elicited with antigen binding to the extracellular CAR domain. The intracellular domain may comprise one or more signaling domains.

[00128] The term "effector function" refers to a specialized function of an immune effector cell. Effector function of the T cell, for example, may be cytolytic activity or help or activity including the secretion of a cytokine. Thus, the term "signaling domain" refers to the portion of a protein which transduces the effector function signal and that directs the cell to perform a specialized function. While usually the entire signaling domain can be employed, in many cases it is not necessary to use the entire domain. To the extent that a truncated portion of a signaling domain is used, such truncated portion may be used in place of the entire domain as long as it transduces the effector function signal. The term signaling domain is meant to include any truncated portion of the signaling domain sufficient to transducing effector function signal. In some embodiments, the signaling domain of a CAR comprises one or more of an intracellular signaling portion of human CD3 zeta, CD28, CD137, TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta or CD3 epsilon.

[00129] In some cases, signals generated through the TCR alone may be insufficient for full activation of the effector cell (e.g., T cell) and that a secondary or co-stimulatory signal may also be required. Thus, effector cell (e.g., T cell) activation can be said to be mediated by two distinct classes of intracellular signaling domains: signaling domains that initiate antigen-dependent primary activation through the TCR (*e.g.*, a TCR/CD3 complex) and co-stimulatory domains that act in an antigen-independent manner to provide a secondary or co-stimulatory signal. In some embodiments, a CAR comprises an intracellular domain that comprises one or more "co-stimulatory domains" and a "signaling domain."

[00130] Signaling domains regulate primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs. Illustrative examples of ITAM containing signaling domains that are useful in particular embodiments include those derived from FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d. In some embodiments, an anti-HLA-G CAR comprises a CD3ζ signaling domain and one or more co-stimulatory domains. The intracellular signaling and co-stimulatory domains may be linked in any order in tandem to the carboxyl terminus of the transmembrane domain. In some embodiments, the intracellular domain comprises a CD3ζ signaling domain amino acid sequence comprising, consisting of, or consisting essentially of, the sequence of SEQ ID NO: 74.

[00131] In some embodiments, CARs comprise one or more co-stimulatory domains to enhance the efficacy and expansion of T cells expressing CAR receptors. As used herein, the term "co-stimulatory domain", refers to an intracellular domain of a co-stimulatory molecule. Co-stimulatory molecules are cell surface molecules other than antigen receptors or Fc receptors that provide a second signal required for efficient activation and function of T lymphocytes upon binding to antigen. Illustrative examples of such co-stimulatory molecules include TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD40LG (CD40L), CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DAP10, DAP-12, ITGB2 (LFA-1), LAT, MyD88, NKD2C (KLRC2), SLP76, TNFRS18 (GITR), TNFRSF14 (HVEM), TRIM, and ZAP70. In one embodiment, a CAR comprises one or more co-stimulatory domains selected from CD28 and CD137, and a CD3ζ signaling domain.

[00132] In some embodiments, the intracellular domain comprises one or more co-stimulatory domains selected from CD28 and 4-1BB. Signaling via CD28 is required for IL2 production and proliferation, but does not play a primary role in sustaining T cell function and activity. 4-1BB (a tumor necrosis factor-receptor family member expressed following CD28 activation) and OX-40 are involved in driving long-term survival of T cells, and accumulation of T cells. The ligands for these receptors typically are expressed on professional antigen presenting cells such as dendritic cells and activated macrophages, but not on tumor cells.

[00133] In some embodiments, the intracellular domain of a CAR comprises a CD28 co-stimulatory domain and a CD3ζ signaling domain. In some embodiments, the intracellular domain of a CAR comprises a 4-1BB co-stimulatory domain and a CD3ζ signaling domain.

In some embodiments, the CD28, 4-1BB, and CD3ζ domains are human. In some embodiments, expressing a CAR that incorporates CD28 and/or 4-1BB signaling domains in $CD4^+$ T cells enhances the activity and anti-tumor potency of those cells compared to those expressing a CAR that contains only the CD3ζ signaling domain. In some embodiments, the anti-HLA-G CARs contain both CD28 and 4-1BB co-stimulatory domains.

[00134] In some embodiments, the intracellular domain comprises a CD28 sequence comprising, consisting of, or consisting essentially of, SEQ ID NO: 72. In some embodiments, the intracellular domain comprises a 4-1BB sequence comprising, consisting of, or consisting essentially of, SEQ ID NO: 73. In some embodiments, the intracellular domain comprises a CD28 co-stimulatory domain and a CD3ζ signaling domain, wherein the CD28 sequence comprises, consists of, or consists essentially of, SEQ ID NO: 72 and wherein the CD3ζ sequence comprises, consists of, or consists essentially of, SEQ ID NO: 74. In some embodiments, the intracellular domain comprises a 4-1BB co-stimulatory domain and a CD3ζ signaling domain, wherein the 4-1BB sequence comprises, consists of, or consists essentially of, SEQ ID NO: 73, and wherein the CD3ζ sequence comprises, consists of, or consists essentially of, SEQ ID NO: 74.

[00135] The third generation of CARs is based on combining two or more costimulatory sequences (such as 4-1BB-CD28-CD3ζ). These receptors secrete a broader range of cytokines (including TNFα, GM-CSF, and IFNγ), are less susceptible to activation-induced cell death, and show higher efficacy in tumor elimination in mouse models. One or multiple endodomains may be employed, as so-called third generation CARs have at least 2 or 3 signaling domains fused together for additive or synergistic effect, for example.

[00136] The CAR of the disclosure may be a first generation, a second generation, or a third generation CAR as described hereabove. Preferably, the CAR is a second or third generation CAR. Even more preferably, the CAR is a third generation CAR when expressed by a T cell and a first generation CAR when expressed by a NK or a NKT cell.

Exemplary CAR Constructs

[00137] In some embodiments, the anti-HLA-G CAR constructs provided herein comprise an anti-HLA-G binding domain, a hinge domain, a transmembrane domain, and an intracellular domain. In some embodiments, the intracellular domain comprises a signaling domain. In some embodiments, the intracellular domain comprises a signaling domain and a costimulatory domain. In some embodiments, the anti-HLA-G CAR constructs provided herein comprise, in

amino-terminal to carboxyl-terminal order, a scFv that specifically binds to human HLA-G, a spacer, a transmembrane domain, a costimulatory domain and a signaling domain.

[00138] In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain, comprising HCDR1, HCDR2, and HCDR3, and an immunoglobulin VL chain comprising LCDR1, LCDR2, and LCDR3, wherein the HCDR1 comprises an amino acid sequence of SEQ ID NO: 11; the HCDR2 comprises an amino acid sequence of SEQ ID NO: 13; and the HCDR3 comprises an amino acid sequence of SEQ ID NO: 15; and wherein the LCDR1 comprises an amino acid sequence of SEQ ID NO: 37; the LCDR2 comprises an amino acid sequence of SEQ ID NO: 39; the LCDR3 comprises an amino acid sequence of SEQ ID NO: 41.

[00139] In some embodiments, an anti-HLA-G CAR comprises a heavy chain variable region VH comprising one, two, three, or four heavy chain framework regions (FRs) that have been humanized. In some embodiments, an anti-HLA-G CAR comprises a VH comprising, in order from N-terminus to C-terminus: a humanized heavy chain FR1 as set forth in SEQ ID NO: 10 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 10; a CDR1 as set forth in SEQ ID NO. 11; a humanized heavy chain FR2 as set forth in one of SEQ ID NOs: 12 and 26 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 12 or 26; a CDR2 as set forth in SEQ ID NO: 13; a humanized heavy chain FR3 as set forth in SEQ ID NO: 14 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 14; a CDR3 as set forth in SEQ ID NO: 15; and a humanized heavy chain FR4 as set forth in SEQ ID NO: 16 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 16.

[00140] Exemplary humanized VH chains of the anti-HLA-G binding domains described herein are provided below in Table 1.

Table 1: Nucleotide and amino acid sequences of humanized anti-HLA-G heavy chain variable domains

Domain or Region	Sequence	SEQ ID NO
VH5 Heavy chain variable domain (nucleotide)	cagatccagctggtccagtccggatcggagctgaagaagcct ggagcaagcgtgaaggtgtcatgcaaagcgtccggatacact ttcacgacctacgggatgtcatgggtccgccaagcgcctggt cagggcctggaatggatgggctggatctacacttactcaggt gtgccaacttatgccgacgacctgaccggacggttcgtgttc agcttggacacgtcagtgtcaaccgcttacctgcagatcagc tcgctgaaggccgaggacactgccacctactactgcgcccgg gtcagagacggatattaccggtacgcgatggactactggggg cgcggaaccctcgtcactgtctcgtcg	1
VH5 Heavy chain FR1 (nucleotide)	cagatccagctggtccagtccggatcggagctgaagaagcct ggagcaagcgtgaaggtgtcatgcaaagcgtcc	2

VH5 Heavy chain CDR1 (nucleotide)	ggatacactttcacgacctacgggatgtca	3
VH5 Heavy chain FR2 (nucleotide)	tgggtccgccaagcgcctggtcagggcctggaatggatgggc	4
VH5 Heavy chain CDR2 (nucleotide)	tggatctacacttactcaggtgtgccaacttatgccgacgac ctgaccgga	5
VH5 Heavy chain FR3 (nucleotide)	cggttcgtgttcagcttggacacgtcagtgtcaaccgcttac ctgcagatcagctcgctgaaggccgaggacactgccacctac tactgcgcccgg	6
VH5 Heavy chain CDR3 (nucleotide)	gtcagagacggatattaccggtacgcgatggactac	7
VH5 Heavy chain FR4 (nucleotide)	tgggggcgcggaaccctcgtcactgtctcgtcg	8
VH5 Heavy chain variable domain (amino acid)	QIQLVQSGSELKKPGASVKVSCKASGYTFTTYGMSWVRQAPG QGLEWMGWIYTYSGVPTYADDLTGRFVFSLDTSVSTAYLQIS SLKAEDTATYYCARVRDGYYRYAMDYWGRGTLVTVSS	9
VH5 Heavy chain FR1 (amino acid)	QIQLVQSGSELKKPGASVKVSCKAS	10
VH5 Heavy chain CDR1 (amino acid)	GYTFTTYGMS	11
VH5 Heavy chain FR2 (amino acid)	WVRQAPGQGLEWMG	12
VH5 Heavy chain CDR2 (amino acid)	WIYTYSGVPTYADDLTG	13
VH5 Heavy chain FR3 (amino acid)	RFVFSLDTSVSTAYLQISSLKAEDTATYYCAR	14
VH5 Heavy chain CDR3 (amino acid)	VRDGYYRYAMDY	15
VH5 Heavy chain FR4 (amino acid)	WGRGTLVTVSS	16
VH4 Heavy chain variable domain (nucleotide)	cagatccaactggtgcaatccggcagcgaactgaaaaagcct ggagcgtcggtgaaggtcagctgcaaagcgtccgggtacact ttcaccacgtacggcatgtcatgggtcaggcaggccccgggt cagggcctgaagtggatggggtggatctacacctactcggga gtgcccacttacgcagatgacctcaccggacggtttgtcttt tcactggatacctccgtctcaaccgcctacctgcaaatctcg tcgctgaaggccgaagacaccgctacgtactactgtgcacgg gtcagggacggctactaccggtatgctatggattactgggga agaggaacgctcgtgaccgtgtcgtcc	17
VH4 Heavy chain FR1 (nucleotide)	cagatccaactggtgcaatccggcagcgaactgaaaaagcct ggagcgtcggtgaaggtcagctgcaaagcgtcc	18
VH4 Heavy chain CDR1 (nucleotide)	gggtacactttcaccacgtacggcatgtca	19
VH4 Heavy chain FR2 (nucleotide)	tgggtcaggcaggccccgggtcagggcctgaagtggatgggg	20
VH4 Heavy chain CDR2 (nucleotide)	tggatctacacctactcgggagtgcccacttacgcagatgac ctcaccgga	21
VH4 Heavy chain FR3 (nucleotide)	cggtttgtcttttcactggatacctccgtctcaaccgcctac ctgcaaatctcgtcgctgaaggccgaagacaccgctacgtac tactgtgcacgg	22
VH4 Heavy chain CDR3 (nucleotide)	gtcagggacggctactaccggtatgctatggattac	23
VH4 Heavy chain FR4 (nucleotide)	tggggaagaggaacgctcgtgaccgtgtcgtcc	24

VH4 Heavy chain variable domain (amino acid)	QIQLVQSGSELKKPGASVKVSCKASGYTFTTYGMSWVRQAPG QGLKWMGWIYTYSGVPTYADDLTGRFVFSLDTSVSTAYLQIS SLKAEDTATYYCARVRDGYYRYAMDYWGRGTLVTVSS	25
VH4 Heavy chain FR1 (amino acid)	QIQLVQSGSELKKPGASVKVSCKAS	10
VH4 Heavy chain CDR1 (amino acid)	GYTFTTYGMS	11
VH4 Heavy chain FR2 (amino acid)	WVRQAPGQGLKWMG	26
VH4 Heavy chain CDR2 (amino acid)	WIYTYSGVPTYADDLTG	13
VH4 Heavy chain FR3 (amino acid)	RFVFSLDTSVSTAYLQISSLKAEDTATYYCAR	14
VH4 Heavy chain CDR3 (amino acid)	VRDGYYRYAMDY	15
VH4 Heavy chain FR4 (amino acid)	WGRGTLVTVSS	16

In the variable domains, CDR1, CDR2 and CDR3 (from left to right) sequences are underlined.

[00141] In some embodiments, an anti-HLA-G CAR comprises a light chain variable region comprising one, two, three, or four light chain FRs that have been humanized. In some embodiments, an anti-HLA-G CAR comprises a light chain variable region comprising, in order from N-terminus to C-terminus: a humanized light chain FR1 as set forth in SEQ ID NO: 36 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 36; a CDR1 as set forth in SEQ ID NO: 37; a humanized light chain FR2 as set forth in SEQ ID NO: 38 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 38; a CDR2 as set forth in SEQ ID NO: 39; a humanized light chain FR3 as set forth in one of SEQ ID NOs: 40 and 51 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 40 or 51; a CDR3 as set forth in SEQ ID NO: 41; and a humanized light chain FR4 as set forth in SEQ ID NO: 42 or at least 80, 85, 90 or 95% of identity with SEQ ID NO: 42.

[00142] Exemplary humanized VL chains of the anti-HLA-G binding domains described herein are provided below in Table 2.

Table 2. Nucleotide and amino acid sequences of humanized anti-HLA-G light chain variable domains

Domain or Region	Sequence	SEQ ID NO
VL5 Light chain variable domain (nucleotide)	gatattcagatgacccagagcccatcatcgctgagcgtc tcggtgggagatcgggtgactatcacttgcaaggcgtca caagacatcaaccgctacatcgcctggtatcagcagcag cctggaaaggctcctaagctgctgattcacttcacttca accctccaatcgggagtccctagccgctttagcggatca ggttccggcaccgatttcactttcactatttcatcgctc cagccggaggacatcgcaacctactactgccttcagtac gatgacctgcgcaccttcggacagggcacgaaactggag attaaa	27

VL5 Light chain FR1 (nucleotide)	gatattcagatgacccagagcccatcatcgctgagcgtc tcggtgggagatcgggtgactatcacttgc	28
VL5 Light chain CDR1 (nucleotide)	aaggcgtcacaagacatcaaccgctacatcgcc	29
VL5 Light chain FR2 (nucleotide)	tggtatcagcagcagcctggaaaggctcctaagctgctg attcac	30
VL5 Light chain CDR2 (nucleotide)	ttcacttcaaccctccaatcg	31
VL5 Light chain FR3 (nucleotide)	ggagtccctagccgctttagcggatcaggttccggcacc gatttcactttcactatttcatcgctccagccggaggac atcgcaacctactactgc	32
VL5 Light chain CDR3 (nucleotide)	ctgcagtatgatgacctacggact	33
VL5 Light chain FR4 (nucleotide)	ttcggacagggcacgaaactggagattaaa	34
VL5 Light chain variable domain (amino acid)	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQ PGKAPKLLIHFTSTLQSGVPSRFSGSGSGTDFTFTISSL QPEDIATYYCLQYDDLRTFGQGTKLEIK	35
VL5 Light chain FR1 (amino acid)	DIQMTQSPSSLSVSVGDRVTITC	36
VL5 Light chain CDR1 (amino acid)	KASQDINRYIA	37
VL5 Light chain FR2 (amino acid)	WYQQQPGKAPKLLIH	38
VL5 Light chain CDR2 (amino acid)	FTSTLQS	39
VL5 Light chain FR3 (amino acid)	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	40
VL5 Light chain CDR3 (amino acid)	LQYDDLRT	41
VL5 Light chain FR4 (amino acid)	FGQGTKLEIK	42
VL4 Light chain variable domain (nucleotide)	gacatccagatgactcagtcaccctcgtcactgtcggtgtcg gtcggagatcgcgtgaccattacttgcaaggcatcccaggac atcaacagatacatcgcgtggtatcaacagcagcctggtaaa gcgccgaagctgctcatccacttcacctccactctacagtca ggcgtgccctcgcgcttcagcggatcaggaagcgggagggac ttcacgtttaccatctcatcccttcagccggaggacatcgcg acctactactgcctgcagtatgatgacctacggacttcggg caaggaaccaaacttgagatcaag	43
VL4 Light chain FR1 (nucleotide)	gacatccagatgactcagtcaccctcgtcactgtcggtgtcg gtcggagatcgcgtgaccattacttgc	44
VL4 Light chain CDR1 (nucleotide)	aaggcatcccaggacatcaacagatacatcgcg	45
VL4 Light chain FR2 (nucleotide)	tggtatcaacagcagcctggtaaagcgccgaagctgctcatc cac	46
VL4 Light chain CDR2 (nucleotide)	ttcacctccactctacagtca	47
VL4 Light chain FR3 (nucleotide)	ggcgtgccctcgcgcttcagcggatcaggaagcgggagggac ttcacgtttaccatctcatcccttcagccggaggacatcgcg acctactactgc	48
VL4 Light chain CDR3 (nucleotide)	ctgcagtatgatgacctacggact	33

VL4 Light chain FR4 (nucleotide)	ttcgggcaaggaaccaaacttgagatcaag	49
VL4 Light chain variable domain (amino acid)	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQPGK APKLLIHFTSTLQSGVPSRFSGSGSGRDFTFTISSLQPEDIA TYYCLQYDDLRTFGQGTKLEIK	50
VL4 Light chain FR1 (amino acid)	DIQMTQSPSSLSVSVGDRVTITC	36
VL4 Light chain CDR1 (amino acid)	KASQDINRYIA	37
VL4 Light chain FR2 (amino acid)	WYQQQPGKAPKLLIH	38
VL4 Light chain CDR2 (amino acid)	FTSTLQS	39
VL4 Light chain FR3 (amino acid)	GVPSRFSGSGSGRDFTFTISSLQPEDIATYYC	51
VL4 Light chain CDR3 (amino acid)	LQYDDLRT	41
VL4 Light chain FR4 (amino acid)	FGQGTKLEIK	42
VL3 Light chain variable domain (nucleotide)	gacatccagatgactcagagcccatcgagcctgtccgtcagc gtgggcgaccgcgtgacgattacttgcaaggcgagccaggac atcaatcgctacatcgcgtggtatcagcaacagcccggaaag gctccaaagctgctgattcacttcacctcgactctgcagtcg ggcatcccgtcaagattctcaggatcaggatcagggcgggat ttcacctttactatttcgagcctgcagccggaggacatcgcg acttactattgcttgcagtacgatgacttgcggaccttcggc cagggaactaagctggagatcaag	52
VL3 Light chain FR1 (nucleotide)	gacatccagatgactcagagcccatcgagcctgtccgtcagc gtgggcgaccgcgtgacgattacttgc	53
VL3 Light chain CDR1 (nucleotide)	aaggcgagccaggacatcaatcgctacatcgcg	54
VL3 Light chain FR2 (nucleotide)	tggtatcagcaacagcccggaaaggctccaaagctgctgatt cac	55
VL3 Light chain CDR2 (nucleotide)	ttcacctcgactctgcagtcg	56
VL3 Light chain FR3 (nucleotide)	ggcatcccgtcaagattctcaggatcaggatcagggcgggat ttcacctttactatttcgagcctgcagccggaggacatcgcg acttactattgc	57
VL3 Light chain CDR3 (nucleotide)	ttgcagtacgatgacttgcggacc	58
VL3 Light chain FR4 (nucleotide)	ttcggccagggaactaagctggagatcaag	59
VL3 Light chain variable domain (amino acid)	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQ PGKAPKLLIHFTSTLQSGIPSRFSGSGSGRDFTFTISSL QPEDIATYYCLQYDDLRTFGQGTKLEIK	60
VL3 Light chain FR1 (amino acid)	DIQMTQSPSSLSVSVGDRVTITC	36
VL3 Light chain CDR1 (amino acid)	KASQDINRYIA	37
VL3 Light chain FR2 (amino acid)	WYQQQPGKAPKLLIH	38
VL3 Light chain CDR2 (amino acid)	FTSTLQS	39

VL3 Light chain FR3 (amino acid)	GIPSRFSGSGSGRDFTFTISSLQPEDIATYYC	61
VL3 Light chain CDR3 (amino acid)	LQYDDLRT	41
VL3 Light chain FR4 (amino acid)	FGQGTKLEIK	42

In the variable domains, CDR1, CDR2 and CDR3 (from left to right) sequences are underlined.

[00143] In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 25 and an immunoglobulin VL chain comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 35. In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain comprising an amino acid sequence that is 100% identical to SEQ ID NO: 25 and an immunoglobulin VL chain comprising an amino acid sequence that is 100% identical to SEQ ID NO: 35. In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain consisting of, or consisting essentially of, the amino acid sequence of SEQ ID NO: 25 and an immunoglobulin VL chain consisting of, or consisting essentially of, the amino acid sequence of SEQ ID NO: 35.

[00144] In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9 and an immunoglobulin VL chain comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 50. In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain comprising an amino acid sequence that is 100% identical to SEQ ID NO: 9 and an immunoglobulin VL chain comprising an amino acid sequence that is 100% identical to SEQ ID NO: 50. In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain consisting of, or consisting essentially of, the amino acid sequence of SEQ ID NO: 9 and an immunoglobulin VL chain consisting of, or consisting essentially of, the amino acid sequence of SEQ ID NO: 50.

[00145] In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9 and an immunoglobulin VL chain comprising an amino acid sequence that is at least 75%, at least

80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 60. In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain comprising an amino acid sequence that is 100% identical to SEQ ID NO: 9 and an immunoglobulin VL chain comprising an amino acid sequence that is 100% identical to SEQ ID NO: 60. In some embodiments, the anti-HLA-G binding domain comprises an immunoglobulin VH chain consisting of, or consisting essentially of, the amino acid sequence of SEQ ID NO: 9 and an immunoglobulin VL chain consisting of, or consisting essentially of, the amino acid sequence of SEQ ID NO: 60.

[00146] Exemplary VH and VL sequences of the anti-HLA-G binding domains described herein are provided in Tables 1 and 2.

[00147] In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 63. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv comprising an amino acid sequence that is 100% identical to SEQ ID NO: 63. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv consisting of, or consisting essentially of, the amino acid sequence of SEQ ID NO: 63.

[00148] In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 65. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv comprising an amino acid sequence that is 100% identical to SEQ ID NO: 65. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv consisting of, or consisting essentially of, the amino acid sequence of SEQ ID NO: 65.

[00149] In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 67. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv comprising an amino acid sequence that is 100% identical to SEQ ID NO: 67. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv consisting of, or consisting essentially of, the amino acid sequence of SEQ ID NO: 67.

[00150] Exemplary scFv sequences of the anti-HLA-G binding domains described herein are provided below in Table 3.

Table 3: Exemplary anti-HLA-G scFv sequences

Domain or Region	Sequence	SEQ ID NO
scFv VH5/VL4 (nucleotide)	gacatccagatgactcagtcaccctcgtcactgtcggtgtcgg tcggagatcgcgtgaccattacttgcaaggcatcccaggacat caacagatacatcgcgtggtatcaacagcagcctggtaaagcg ccgaagctgctcatccacttcacctccactctacagtcaggcg tgccctcgcgcttcagcggatcaggaagcgggagggacttcac gtttaccatctcatcccttcagccggaggacatcgcgacctac tactgcctgcagtatgatgacctacggacttcgggcaaggaa ccaaacttgagatcaag**ggaggaggaggctccggcggaggagg ctctggcggcggcggcagc**cagatccagctggtccagtccgga tcggagctgaagaagcctggagcaagcgtgaaggtgtcatgca aagcgtccggatacactttcacgacctacgggatgtcatgggt ccgccaagcgcctggtcagggcctggaatggatgggctggatc tacacttactcaggtgtgccaacttatgccgacgacctgaccg gacggttcgtgttcagcttggacacgtcagtgtcaaccgctta cctgcagatcagctcgctgaaggccgaggacactgccacctac tactgcgcccgggtcagagacggatattaccggtacgcgatgg actactgggggcgcggaaccctcgtcactgtctcgtcg	62
scFv VH5/VL4 (amino acid)	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQPGKA PKLLIHFTSTLQSGVPSRFSGSGSGRDFTFTISSLQPEDIATY YCLQYDDLRTFGQGTKLEIK**GGGGSGGGGSGGGGS**QIQLVQSG SELKKPGASVKVSCKASGYTFTTYGMSWVRQAPGQGLEWMGWI YTYSGVPTYADDLTGRFVFSLDTSVSTAYLQISSLKAEDTATY YCARVRDGYYRYAMDYWGRGTLVTVSS	63
scFv VH4/VL5 (nucleotide)	gatattcagatgacccagagcccatcatcgctgagcgtctcgg tgggagatcgggtgactatcacttgcaaggcgtcacaagacat caaccgctacatcgcctggtatcagcagcagcctggaaaggct cctaagctgctgattcacttcacttcaaccctccaatcgggag tccctagccgctttagcggatcaggttccggcaccgatttcac tttcactatttcatcgctccagccggaggacatcgcaacctac tactgccttcagtacgatgacctgcgcaccttcggacagggca cgaaactggagattaaa**ggaggaggaggctccggcggaggagg ctctggcggcggcggcagc**cagatccaactggtgcaatccggc agcgaactgaaaaagcctggagcgtcggtgaaggtcagctgca aagcgtccgggtacactttcaccacgtacggcatgtcatgggt caggcaggccccgggtcagggcctgaagtggatggggtggatc tacacctactcgggagtgcccacttacgcagatgacctcaccg gacggtttgtcttttcactggatacctccgtctcaaccgccta cctgcaaatctcgtcgctgaaggccgaagacaccgctacgtac tactgtgcacgggtcagggacggctactaccggtatgctatgg attactggggaagaggaacgctcgtgaccgtgtcgtcc	64
scFv VH4/VL5 (amino acid)	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQPGKA PKLLIHFTSTLQSGVPSRFSGSGSGTDFTFTISSLQPEDIATY YCLQYDDLRTFGQGTKLEIK**GGGGSGGGGSGGGGS**QIQLVQSG SELKKPGASVKVSCKASGYTFTTYGMSWVRQAPGQGLKWMGWI	65

	YTYSGVPTYADDLTGRFVFSLDTSVSTAYLQISSLKAEDTATY YCARVRDGYYRYAMDYWGRGTLVTVSS	
scFv VH5/VL3 (nucleotide)	gacatccagatgactcagagcccatcgagcctgtccgtcagcg tgggcgaccgcgtgacgattacttgcaaggcgagccaggacat caatcgctacatcgcgtggtatcagcaacagcccggaaaggct ccaaagctgctgattcacttcacctcgactctgcagtcgggca tcccgtcaagattctcaggatcaggatcagggcgggatttcac ctttactatttcgagcctgcagccggaggacatcgcgacttac tattgcttgcagtacgatgacttgcggacgttcggccagggaa ctaagctggagatcaag**ggaggaggaggctccggcggaggaggg ctctggcggcggcggcagc**cagatccagctggtccagtccgga tcggagctgaagaagcctggagcaagcgtgaaggtgtcatgca aagcgtccggatacactttcacgacctacgggatgtcatgggt ccgccaagcgcctggtcagggcctggaatggatgggctggatc tacacttactcaggtgtgccaacttatgccgacgacctgaccg gacggttcgtgttcagcttggacacgtcagtgtcaaccgctta cctgcagatcagctcgctgaaggccgaggacactgccacctac tactgcgcccgggtcagagacggatattaccggtacgcgatgg actactgggggcgcggaaccctcgtcactgtctcgtcg	66
scFv VH5/VL3 (amino acid)	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQPGKA PKLLIHFTSTLQSGIPSRFSGSGSGRDFTFTISSLQPEDIATY YCLQYDDLRTFGQGTKLEIK**GGGGSGGGGSGGGGS**QIQLVQSG SELKKPGASVKVSCKASGYTFTTYGMSWVRQAPGQGLEWMGWI YTYSGVPTYADDLTGRFVFSLDTSVSTAYLQISSLKAEDTATY YCARVRDGYYRYAMDYWGRGTLVTVSS	67

In the variable domains, VLCDR1, VLCDR2, VLCDR3, VHCDR1, VHCDR2 and VHCDR3 (from left to right) sequences are underlined; the linkers are bolded.

[00151] In some embodiments, the anti-HLA-G CAR constructs provided herein comprise an anti-HLA-G binding domain, a CD8α hinge domain, a CD8α transmembrane domain, and an intracellular domain comprising a CD3ζ signaling domain. In some embodiments, anti-HLA-G binding domain is a scFv domain comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to one of SEQ ID NOs: 63, 65 and 67. In some embodiments, anti-HLA-G binding domain is a scFv domain comprising an amino acid sequence that is 100% identical to one of SEQ ID NOs: 63, 65 and 67. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv consisting of, or consisting essentially of, the amino acid sequence of one of SEQ ID NOs: 63, 65 and 67.

[00152] In some embodiments, the anti-HLA-G CAR constructs provided herein comprise an anti-HLA-G binding domain, a CD8α hinge domain, a CD8α transmembrane domain, and an intracellular domain comprising a CD3ζ signaling domain and a 4-1BB costimulatory domain. In some embodiments, anti-HLA-G binding domain is a scFv domain comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least

98%, or at least 99% identical to one of SEQ ID NOs: 63, 65 and 67. In some embodiments, anti-HLA-G binding domain is a scFv domain comprising an amino acid sequence that is 100% identical to one of SEQ ID NOs: 63, 65 and 67. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv consisting of, or consisting essentially of, the amino acid sequence of one of SEQ ID NOs: 63, 65 and 67.

[00153] In some embodiments, the anti-HLA-G CAR constructs provided herein comprise an anti-HLA-G binding domain, a CD28 hinge domain, a CD28 transmembrane domain, and an intracellular domain comprising a CD3ζ signaling domain. In some embodiments, anti-HLA-G binding domain is a scFv domain comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to one of SEQ ID NOs: 63, 65 and 67. In some embodiments, anti-HLA-G binding domain is a scFv domain comprising an amino acid sequence that is 100% identical to one of SEQ ID NOs: 63, 65 and 67. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv consisting of, or consisting essentially of, the amino acid sequence of one of SEQ ID NOs: 63, 65 and 67.

[00154] In some embodiments, the anti-HLA-G CAR constructs provided herein comprise an anti-HLA-G binding domain, a CD28 hinge domain, a CD28 transmembrane domain, and an intracellular domain comprising a CD3ζ signaling domain and a CD28 costimulatory domain. In some embodiments, anti-HLA-G binding domain is a humanized scFv domain comprising an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to one of SEQ ID NOs: 63, 65 and 67. In some embodiments, anti-HLA-G binding domain is a scFv domain comprising an amino acid sequence that is 100% identical to one of SEQ ID NOs: 63, 65 and 67. In some embodiments, the anti-HLA-G binding domain is an anti-HLA-G scFv consisting of, or consisting essentially of, the amino acid sequence of one of SEQ ID NOs: 63, 65 and 67.

[00155] An exemplary nucleotide sequence for a CAR construct comprising a CD28 and 4-1BB costimulatory sequence and a CD3 signaling sequence is provided below in Table 4. The general design of the constructs shown in Table 4 is: Ig Kappa signal peptide – humanized anti-HLA-G scFv – hIgG4 hinge domain – CD28 and 4-1BB costimulatory domain – CD3ζ signaling domain. In one embodiment, a CAR construct comprise a truncated CD19 sequence linked via the P2A sequence to the C terminal end of the CAR construct. In some embodiments, the anti-HLA-G CAR construct comprises a nucleotide sequence that is at least 75%, at least

80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 68.

Table 4: Exemplary CAR construct

Domain or Region	Sequence	SEQ ID NO
humanized anti-HLA-G CAR	ATGAAACTGCCCGTGAGACTGCTGGTGCTGATGTTTTGGATTC CCGCTTCCTCGAGCGatattcagatgacccagagcccatcatc gctgagcgtctcggtgggagatcgggtgactatcacttgcaag gcgtcacaagacatcaaccgctacatcgcctggtatcagcagc agcctggaaaggctcctaagctgctgattcacttcacttcaac cctccaatcgggagtccctagccgctttagcggatcaggttcc ggcaccgatttcactttcactatttcatcgctccagccggagg acatcgcaacctactactgccttcagtacgatgaacctgcgcac cttcggacagggcacgaaactggagattaaaggaggaggaggc tccggcggaggaggctctggcggcggcggcagccagatccaac tggtgcaatccggcagcgaactgaaaaagcctggagcgtcggt gaaggtcagctgcaaagcgtccgggtacactttcaccacgtac ggcatgtcatgggtcaggcaggccccgggtcagggcctgaagt ggatggggtggatctacacctactcgggagtgcccacttacgc agatgacctcaccggacggtttgtcttttcactggatacctcc gtctcaacgcctacctgcaaatctcgtcgctgaaggccgaag acaccgctacgtactactgtgcacgggtcagggacggctacta ccggtatgctatggattactggggaagaggaacgctcgtgacc gtgtcgtccGAGAGCAAGTACGGCCCACCCTGCCCTCCATGTC CATTTTGGGTGCTGGTGGTGGTGGGAGGCGTGCTGGCCTGTTA TTCTCTGCTGGTGACCGTGGCCTTCATCATCTTTTGGGTGCGC AGCAAGCGGAGCCGGCTGCTGCACTCCGACTACATGAACATGA CCCCAAGACGGCCCGGACCAACAAGGAAGCACTACCAGCCTTA TGCACCACCCCGCGATTTTGCAGCATACCGGAGCAAGAGAGGC AGGAAGAAGCTGCTGTATATCTTCAAGCAGCCCTTCATGCGGC CCGTGCAGACCACACAGGAGGAGGACGGCTGCTCCTGTAGGTT CCCTGAAGAGGAGGAGGGAGGATGCGAGCTGAGAGTGAAGTTT TCTAGGAGCGCCGATGCACCAGCATACCAGCAGGGACAGAATC AGCTGTATAACGAGCTGAATCTGGGCAGGCGCGAGGAGTACGA CGTGCTGGATAAGAGGAGAGGACGGGACCCCGAGATGGGAGGC AAGCCAAGGCGCAAGAACCCCCAGGAGGGCCTGTACAATGAGC TGCAGAAGGACAAGATGGCCGAGGCCTATAGCGAGATCGGCAT GAAGGGAGAGCGGAGAAGGGGCAAGGGACACGATGGCCTGTAC CAGGGCCTGTCCACCGCCACAAAGGACACCTATGATGCCCTGC ACATGCAGGCCCTGCCTCCAAGGGGCAGCGGAGCCACAAACTT CTCCCTGCTGAAGCAGGCCGGCGATGTGGAGGAGAATCCTGGA CCAATGCCACCTCCAAGGCTGCTGTTCTTTCTGCTGTTTCTGA CCCCAATGGAGGTGCGGCCAGAGGAGCCACTGGTGGTGAAGGT GGAGGAGGGCGACAACGCCGTGCTGCAGTGTCTGAAGGGCACA AGCGATGGCCCTACCCAGCAGCTGACATGGTCTAGAGAGAGCC CTCTGAAGCCATTCCTGAAGCTGTCCCTGGGCCTGCCAGGCTT AGGAATCCATATGAGGCCCCTGGCTATCTGGCTGTTTATCTTC AACGTGAGCCAGCAGATGGGAGGCTTCTATCTGTGCCAGCCAG GACCACCTAGCGAGAAGGCATGGCAGCCTGGATGGACCGTGAA CGTGGAGGGCAGCGGAGAGCTGTTTCGCTGGAACGTGAGCGAC	68

	CTGGGAGGCCTGGGATGTGGCCTGAAGAACCGGAGCTCCGAGG GACCCTCTAGCCCTAGCGGCAAGCTGATGTCCCCAAAGCTGTA CGTGTGGGCCAAGGATAGACCAGAGATCTGGGAGGGAGAGCCA CCATGCCTGCCTCCAAGGGACAGCCTGAATCAGTCCCTGTCTC AGGATCTGACCATGGCCCCCGGCTCTACACTGTGGCTGAGCTG TGGAGTGCCACCTGACAGCGTGTCCCGGGGCCCTCTGAGCTGG ACCCACGTGCACCCAAAGGGCCCCAAGTCCCTGCTGTCTCTGG AGCTGAAGGACGATCGCCCTGCCCGGGACATGTGGGTCATGGA GACAGGCCTGCTGCTGCCACGCGCCACAGCACAGGATGCCGGC AAGTACTATTGCCACCGGGGCAACCTGACCATGAGCTTCCACC TGGAGATCACAGCCAGACCCGTGCTGTGGCACTGGCTGCTGAG GACCGGAGGATGGAAGGTGTCCGCCGTGACCCTGGCATACCTG ATTTTCTGTCTGTGCTCCCTGGTGGGCATCCTGCACCTGTGA	
CAR Ig Kappa Signal peptide	ATGAAACTGCCCGTGAGACTGCTGGTGCTGATGTTTTGGATTC CCGCTTCCTCGAGC	69
CAR scFv (VH4/VL5)	Gatattcagatgacccagagcccatcatcgctgagcgtctcgg tgggagatcgggtgactatcacttgcaaggcgtcacaagacat caaccgctacatcgcctggtatcagcagcagcctggaaaggct cctaagctgctgattcacttcacttcaaccctccaatcgggag tccctagccgctttagcggatcaggttccggcaccgatttcac tttcactatttcatcgctccagccggaggacatcgcaacctac tactgccttcagtacgatgacctgcgcaccttcggacagggca cgaaactggagattaaaggaggaggaggctccggcggaggagg ctctggcggcggcggcagccagatccaactggtgcaatccggc agcgaactgaaaaagcctggagcgtcggtgaaggtcagctgca aagcgtccgggtacactttcaccacgtacggcatgtcatgggt caggcaggccccgggtcagggcctgaagtggatggggtggatc tacacctactcgggagtgcccacttacgcagatgacctcaccg gacggtttgtcttttcactggatacctccgtctcaaccgccta cctgcaaatctcgtcgctgaaggccgaagacaccgctacgtac tactgtgcacgggtcagggacggctactaccggtatgctatgg attactggggaagaggaacgctcgtgaccgtgtcgtcc	64
CAR scFv linker	ggaggaggaggctccggcggaggaggctctggcggcggcggca gc	70
CAR VLCDR1	aaggcgtcacaagacatcaaccgctacatcgcc	29
CAR VLCDR2	ttcacttcaaccctccaatcg	31
CAR VLCDR3	cttcagtacgatgacctgcgcacc	33
CAR VHCDR1	gggtacactttcaccacgtacggcatgtca	19
CAR VHCDR2	tggatctacacctactcgggagtgcccacttacgcagatgacc tcaccgga	21
CAR VHCDR3	gtcagggacggctactaccggtatgctatggattact	23
CAR hIgG4 Hinge	GAGAGCAAGTACGGCCCACCCTGCCCTCCATGTCCA	71
CD28	TTTTGGGTGCTGGTGGTGGTGGGAGGCGTGCTGGCCTGTTATT CTCTGCTGGTGACCGTGGCCTTCATCATCTTTTGGGTGCGCAG CAAGCGGAGCCGGCTGCTGCACTCCGACTACATGAACATGACC CCAAGACGGCCCGGACCAACAAGGAAGCACTACCAGCCTTATG CACCACCCCGCGATTTTGCAGCATACCGGAGC	72
4-1BB	AAGAGAGGCAGGAAGAAGCTGCTGTATATCTTCAAGCAGCCCT TCATGCGGCCCGTGCAGACCACACAGGAGGAGGACGGCTGCTC CTGTAGGTTCCCTGAAGAGGAGGAGGGAGGATGCGAGCTG	73
CD3 zeta	AGAGTGAAGTTTTCTAGGAGCGCCGATGCACCAGCATACCAGC AGGGACAGAATCAGCTGTATAACGAGCTGAATCTGGGCAGGCG	74

	CGAGGAGTACGACGTGCTGGATAAGAGGAGAGGACGGGACCCC GAGATGGGAGGCAAGCCAAGGCGCAAGAACCCCCAGGAGGGCC TGTACAATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTATAG CGAGATCGGCATGAAGGGAGAGCGGAGAAGGGGCAAGGGACAC GATGGCCTGTACCAGGGCCTGTCCACCGCCACAAAGGACACCT ATGATGCCCTGCACATGCAGGCCCTGCCTCCAAGGGGCAGCGG A	
CAR cleavable linker porcine teschovirus-1 2A (P2A)	GCCACAAACTTCTCCCTGCTGAAGCAGGCCGGCGATGTGGAGG AGAATCCTGGACCA	75
CAR truncated human CD19	ATGCCACCTCCAAGGCTGCTGTTCTTTCTGCTGTTTCTGACCC CAATGGAGGTGCGGCCAGAGGAGCCACTGGTGGTGAAGGTGGA GGAGGGCGACAACGCCGTGCTGCAGTGTCTGAAGGGCACAAGC GATGGCCCTACCCAGCAGCTGACATGGTCTAGAGAGAGCCCTC TGAAGCCATTCCTGAAGCTGTCCCTGGGCCTGCCAGGCTTAGG AATCCATATGAGGCCCCTGGCTATCTGGCTGTTTATCTTCAAC GTGAGCCAGCAGATGGGAGGCTTCTATCTGTGCCAGCCAGGAC CACCTAGCGAGAAGGCATGGCAGCCTGGATGGACCGTGAACGT GGAGGGCAGCGGAGAGCTGTTTCGCTGGAACGTGAGCGACCTG GGAGGCCTGGGATGTGGCCTGAAGAACCGGAGCTCCGAGGGAC CCTCTAGCCCTAGCGGCAAGCTGATGTCCCCAAAGCTGTACGT GTGGGCCAAGGATAGACCAGAGATCTGGGAGGGAGAGCCACCA TGCCTGCCTCCAAGGGACAGCCTGAATCAGTCCCTGTCTCAGG ATCTGACCATGGCCCCCGGCTCTACACTGTGGCTGAGCTGTGG AGTGCCACCTGACAGCGTGTCCCGGGGCCCTCTGAGCTGGACC CACGTGCACCCAAAGGGCCCCAAGTCCCTGCTGTCTCTGGAGC TGAAGGACGATCGCCCTGCCCGGGACATGTGGGTCATGGAGAC AGGCCTGCTGCTGCCACGCGCCACAGCACAGGATGCCGGCAAG TACTATTGCCACCGGGGCAACCTGACCATGAGCTTCCACCTGG AGATCACAGCCAGACCCGTGCTGTGGCACTGGCTGCTGAGGAC CGGAGGATGGAAGGTGTCCGCCGTGACCCTGGCATACCTGATT TTCTGTCTGTGCTCCCTGGTGGGCATCCTGCACCTGTGA	76

In the variable domains, VLCDR1, VLCDR2, VLCDR3, VHCDR1, VHCDR2 and VHCDR3 (from left to right) sequences are underlined.

[00156] FIG. 1 is an exemplary schematic design of the CAR structure based on LFTT-1 with the cleavable linker P2A and the truncated hCD19 as reporter. LFTT-1 is described in WO2020043899, which is incorporated herein by reference in its entirety. Truncated CD19 marker is used for identification of CAR-T product. Co-stimulatory domains CD28 and 4-1BB are used for enhanced anti-tumor activity. Internal EF1α promoter is used for optimal CAR expression.

Polypeptides

[00157] In some embodiments, the present disclosure provides anti-HLA-G CAR polypeptides and fragments thereof. In some embodiments, the CAR is an anti-HLA-G CAR comprising a nucleotide sequence as set forth in SEQ ID NO: 68.

[00158] "Polypeptide," "peptide" and "protein" are used interchangeably, unless specified to the contrary, and according to conventional meaning, *i.e.*, as a sequence of amino acids. Polypeptides are not limited to a specific length, *e.g.*, they may comprise a full-length polypeptide or a polypeptide fragment, and may include one or more post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. In some embodiments, the CAR polypeptides comprise a signal (or leader) sequence at the N-terminal end of the protein, which co-translationally or post-translationally directs transfer of the protein. Illustrative examples of suitable signal sequences useful in CARs contemplated in some embodiments include, but are not limited to the IgG1 heavy chain signal polypeptide, a CD8α signal polypeptide, or a human GM-CSFR-α signal polypeptide. Polypeptides can be prepared using any of a variety of well-known recombinant and/or synthetic techniques.

Polypeptide variants

[00159] Polypeptides contemplated herein, encompass the CARs of the present disclosure, as well as functional variants thereof. The term "functional variant" as used herein refers to a CAR, polypeptide, or protein having substantial or significant sequence identity or similarity to a parent CAR, which functional variant retains the biological activity of the CAR of the parent CAR. Functional variants encompass, for example, CAR variants that retain the ability to recognize target cells to a similar extent, the same extent, or to a higher extent, as the parent CAR. Such variants may be naturally occurring or may be synthetically generated, for example, by modifying one or more of the above polypeptide sequences. For example, in some embodiments, it may be desirable to improve the binding affinity and/or other biological properties of the CARs by introducing one or more substitutions, deletions, additions and/or insertions into a binding domain, hinge, TM domain, co-stimulatory domain or signaling domain of a CAR polypeptide. In some embodiments, CAR polypeptides include polypeptides having at least about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 86%, 97%, 98%, or 99% identity to any of the CAR polypeptides described herein (*e.g.*, SEQ ID NO: 68), typically where the variant maintains at least one biological activity of the reference sequence.

[00160] As noted above, in some embodiments, polypeptides may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of a

reference polypeptide can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985, *Proc. Natl. Acad. Sci. USA*. 82: 488-492), Kunkel *et al.*, (1987, *Methods in Enzymol*, 154: 367-382), U.S. Pat. No. 4,873,192, Watson, J. D. *et al.*, (*Molecular Biology of the Gene*, Fourth Edition, Benjamin/Cummings, Menlo Park, Calif., 1987) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.*, (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.).

[00161] A functional variant can, for example, comprise the amino acid sequence of the parent CAR with at least one conservative amino acid substitution. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Modifications may be made in the structure of the polynucleotides and polypeptides contemplated in some embodiments and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics. For instance, the conservative amino acid substitution can be an acidic/negatively charged polar amino acid substituted for another acidic/negatively charged polar amino acid (e.g., Asp or Glu), an amino acid with a nonpolar side chain substituted for another amino acid with a nonpolar side chain (e.g., Ala, Gly, Val, Ile, Leu, Met, Phe, Pro, Trp, Cys, Val, etc.), a basic/positively charged polar amino acid substituted for another basic/positively charged polar amino acid (e.g. Lys, His, Arg, etc.), an uncharged amino acid with a polar side chain substituted for another uncharged amino acid with a polar side chain (e.g., Asn, Gln, Ser, Thr, Tyr, etc.), an amino acid with a beta-branched side-chain substituted for another amino acid with a beta-branched side-chain (e.g., Ile, Thr, and Val), an amino acid with an aromatic side-chain substituted for another amino acid with an aromatic side chain (e.g., His, Phe, Trp, and Tyr), etc. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity can be found using computer programs well known in the art, such as DNASTAR, DNA Strider, Geneious, Mac Vector, or Vector NTI software.

[00162] Alternatively or additionally, the functional variants can comprise the amino acid sequence of the parent CAR with at least one non-conservative amino acid substitution. In this case, it is preferable for the non-conservative amino acid substitution to not interfere with or inhibit the biological activity of the functional variant. The non-conservative amino acid

substitution may enhance the biological activity of the functional variant, such that the biological activity of the functional variant is increased as compared to the parent CAR.

[00163] Polypeptide variants further include glycosylated forms, aggregative conjugates with other molecules, and covalent conjugates with unrelated chemical moieties (*e.g.*, pegylated molecules). Covalent variants can be prepared by linking functionalities to groups which are found in the amino acid chain or at the N- or C-terminal residue, as is known in the art. Variants also include allelic variants, species variants, and muteins. Truncations or deletions of regions which do not affect functional activity of the proteins are also variants.

[00164] In one embodiment, where expression of two or more polypeptides is desired, the polynucleotide sequences encoding them can be separated by and IRES sequence as discussed elsewhere herein. In another embodiment, two or more polypeptides can be expressed as a fusion protein that comprises one or more self-cleaving polypeptide sequences, *e.g.*, a 2A sequence.

Fusion Polypeptides

[00165] Polypeptides contemplated in some embodiments include fusion polypeptides (*e.g.*, a CAR fusion protein). In some embodiments, fusion polypeptides and polynucleotides encoding fusion polypeptides are provided, *e.g.*, CARs. Fusion polypeptides and fusion proteins refer to a polypeptide having at least two, three, four, five, six, seven, eight, nine, or ten or more polypeptide segments. Fusion polypeptides are typically linked C-terminus to N-terminus, although they can also be linked C-terminus to C-terminus, N-terminus to N-terminus, or N-terminus to C-terminus. The polypeptides of the fusion protein can be in any order or a specified order. Fusion polypeptides or fusion proteins can also include conservatively modified variants, polymorphic variants, alleles, mutants, subsequences, and interspecies homologs, so long as the desired transcriptional activity of the fusion polypeptide is preserved. Fusion polypeptides may be produced by chemical synthetic methods or by chemical linkage between the two moieties or may generally be prepared using other standard techniques. Ligated DNA sequences comprising the fusion polypeptide are operably linked to suitable transcriptional or translational control elements as discussed elsewhere herein.

[00166] In one embodiment, a fusion partner comprises a sequence that assists in expressing the protein (an expression enhancer) at higher yields than the native recombinant protein. Other fusion partners may be selected so as to increase the solubility of the protein or to enable the

protein to be targeted to desired intracellular compartments or to facilitate transport of the fusion protein through the cell membrane.

[00167] In some embodiments, fusion polypeptides may further comprise a polypeptide cleavage signal between each of the polypeptide domains described herein. In addition, a polypeptide cleavage site can be put into any linker peptide sequence. Exemplary polypeptide cleavage signals include polypeptide cleavage recognition sites such as protease cleavage sites, nuclease cleavage sites (*e.g.*, rare restriction enzyme recognition sites, self-cleaving ribozyme recognition sites), and self-cleaving viral oligopeptides (see deFelipe and Ryan, 2004. *Traffic*, 5(8); 616-26).

[00168] Suitable protease cleavages sites and self-cleaving peptides are known to the skilled person (*see, e.g.*, in Ryan *et al.*, 1997. *J. Gener. Virol.* 78, 699-722; Scymczak *et al.* (2004) Nature Biotech. 5, 589-594). Exemplary protease cleavage sites include, but are not limited to the cleavage sites of potyvirus NIa proteases (*e.g.*, tobacco etch virus protease), potyvirus HC proteases, potyvirus P1 (P35) proteases, byovirus NIa proteases, byovirus RNA-2-encoded proteases, aphthovirus L proteases, enterovirus 2A proteases, rhinovirus 2A proteases, picorna 3C proteases, comovirus 24K proteases, nepovirus 24K proteases, RTSV (rice tungro spherical virus) 3C-like protease, PYVF (parsnip yellow fleck virus) 3C-like protease, heparin, thrombin, factor Xa, and enterokinase.

[00169] In some embodiments, the self-cleaving polypeptide site comprises a 2A or 2A-like site, sequence or domain (Donnelly *et al.*, 2001. *J. Gen. Virol.* 82:1027-1041). Particularly, the mechanism of 2A-mediated "self-cleavage" was recently discovered to be ribosome skipping the formation of a glycyl-prolyl peptide bond at the C-terminus of the 2A peptide. The 2A-peptide-mediated cleavage commences after the translation. Successful skipping and recommencement of translation results in two "cleaved" proteins: the protein upstream of the 2A is attached to the complete 2A peptide except for the C-terminal proline, and the protein downstream of the 2A is attached to one proline at the N-terminus. Successful skipping but ribosome fall-off and discontinued translation results in only the protein upstream of 2A. Several 2A peptides have been identified in picornaviruses, insect viruses and type C rotaviruses.

[00170] Examples of cleavable linker according to the disclosure include, but are not limited to, porcine teschovirus-1 2A (P2A), FMDV 2A (F2A); equine rhinitis A virus (ERAV) 2A (E2A); and Thosea asigna virus 2A (T2A), cytoplasmic polyhedrosis virus 2A (BmCPV2A)

and flacherie Virus 2A (BmIFV2A), or a combination thereof, for example such as described in Kim et al. (2011) PLoS ONE 6(4): e18556 and in Liu et al (2017) Sci Rep. 2017; 7: 2193.

[00171] Preferably, the cleavable linker is P2A which comprises or consists of the sequence set forth in SEQ ID NO: 75 or a sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of identity therewith.

[00172] In one embodiment, the N-terminus of the cleavable linker is operably linked to the C-terminus of the CAR endodomain and/or the C-terminus of the cleavable linker is operably linked to the N-terminus of a reporter.

Polynucleotides

[00173] In some embodiments, the present disclosure provides polynucleotides or nucleic acid molecules encoding one or more CAR polypeptides. As used herein, the terms "nucleotide" or "nucleic acid" refer to deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and DNA/RNA hybrids. Polynucleotides may be single-stranded or double-stranded and either recombinant, synthetic, or isolated. Polynucleotides include, but are not limited to: pre-messenger RNA (pre-mRNA), messenger RNA (mRNA), RNA, genomic DNA (gDNA), PCR amplified DNA, complementary DNA (cDNA), synthetic DNA, or recombinant DNA. Polynucleotides refer to a polymeric form of nucleotides of at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 100, at least 200, at least 300, at least 400, at least 500, at least 1000, at least 5000, at least 10000, or at least 15000 or more nucleotides in length, either ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide, as well as all intermediate lengths. It will be readily understood that "intermediate lengths," in this context, means any length between the quoted values, such as 6, 7, 8, 9, *etc.*, 101, 102, 103, *etc.*; 151, 152, 153, *etc.*; 201, 202, 203, *etc.*

[00174] In some embodiments, polynucleotides may be codon-optimized. As used herein, the term "codon-optimized" refers to substituting codons in a polynucleotide encoding a polypeptide in order to increase the expression, stability and/or activity of the polypeptide. Factors that influence codon optimization include, but are not limited to one or more of: (i) variation of codon biases between two or more organisms or genes or synthetically constructed bias tables, (ii) variation in the degree of codon bias within an organism, gene, or set of genes, (iii) systematic variation of codons including context, (iv) variation of codons according to their decoding tRNAs, (v) variation of codons according to GC %, either overall or in one position of the triplet, (vi) variation in degree of similarity to a reference sequence for example

a naturally occurring sequence, (vii) variation in the codon frequency cutoff, (viii) structural properties of mRNAs transcribed from the DNA sequence, (ix) prior knowledge about the function of the DNA sequences upon which design of the codon substitution set is to be based, (x) systematic variation of codon sets for each amino acid, (xi) isolated removal of spurious translation initiation sites and/or (xii) elimination of fortuitous polyadenylation sites otherwise leading to truncated RNA transcripts.

[00175] Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity," and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (*i.e.*, only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150 in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison window may comprise additions or deletions (*i.e.*, gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (*i.e.*, resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.*, 1997, Nucl. Acids Res. 25:3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons Inc, 1994-1998, Chapter 15.

[00176] The recitations "sequence identity" or, for example, comprising a "sequence 50% identical to," as used herein, refer to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a

"percentage of sequence identity" may be calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.*, A, T, C, G, I) or the identical amino acid residue (*e.g.*, Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

[00177] As used herein, the terms "polynucleotide variant" and "variant" and the like refer to polynucleotides displaying substantial sequence identity with a reference polynucleotide sequence or polynucleotides that hybridize with a reference sequence under stringent conditions that are defined hereinafter. These terms include polynucleotides in which one or more nucleotides have been added or deleted, or replaced with different nucleotides compared to a reference polynucleotide. In this regard, it is well understood in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference polynucleotide whereby the altered polynucleotide retains the biological function or activity of the reference polynucleotide.

[00178] In some embodiments, polynucleotides or variants have at least or about 50%, 55%, 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%,76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%,85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a reference sequence.

[00179] Moreover, it will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide, or fragment of variant thereof, as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated in some embodiments, for example polynucleotides that are optimized for human and/or primate codon selection. Further, alleles of the genes comprising the polynucleotide sequences provided herein may also be used. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides.

[00180] The polynucleotides contemplated herein, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters and/or enhancers, untranslated regions (UTRs), signal sequences, Kozak sequences, polyadenylation

signals, additional restriction enzyme sites, multiple cloning sites, internal ribosomal entry sites (IRES), recombinase recognition sites (*e.g.*, LoxP, FRT, and Att sites), termination codons, transcriptional termination signals, and polynucleotides encoding self-cleaving polypeptides, epitope tags, as disclosed elsewhere herein or as known in the art, such that their overall length may vary considerably. It is therefore contemplated that a polynucleotide fragment of almost any length may be employed in some embodiments, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

[00181] Provided herein is a nucleic acid construct comprising sequences encoding an external domain, an intracellular domain, a transmembrane, optionally a hinge domain, and optionally a cleavable linker, a reporter and/or a signal peptide as described hereabove. In one embodiment, a nucleic acid construct comprises a nucleotide sequence set forth in SEQ ID NO: 68.

[00182] In one embodiment, the nucleic acid construct sequentially comprises or consists in, from N to C terminus: optionally a peptide signal sequence, an anti-HLA-G antibody or fragment thereof, preferably an anti-HLA-G scFv, a spacer domain, a transmembrane domain, at least one intracellular domain, and optionally a cleavable linker and a reporter. In a yet further embodiment, the nucleic acid construct further comprises a tag, preferably a Flag tag.

[00183] In some embodiments, the nucleic acid construct comprises:

(a) a nucleic acid sequence encoding an anti-HLA-G scFv as described above;

(b) optionally a nucleic acid sequence encoding a hinge, preferably selected from the group consisting of (i) CD28 hinge, (ii) CD8 alpha hinge, (iii) a human IgG4 hinge domain, (iv) a human IgG4 hinge domain and a CH3 human IgG4 domain or (v) a mutated CH2 human IgG4 domain, a human IgG4 hinge domain and a CH3 human IgG4 hinge domain

(c) a nucleic acid sequence encoding a transmembrane domain, preferably a CD28 transmembrane domain;

(d) a nucleic acid sequence encoding an endodomain, preferably a 4-1BB domain and/or a CD3 ζ domain;

(e) optionally a cleavable linker, preferably a P2A cleavable linker;

(f) optionally a reporter, preferably a hCD19t reporter; and/or

(g) optionally a signal peptide, preferably selected from the group consisting of CD8a, a mouse Ig Kappa signal peptide, a human IgG4 signal peptide and an IL2 signal peptide.

[00184] In some embodiments, the nucleic acid construct comprises:

(i) a nucleic acid sequence encoding the VH of SEQ ID NO: 17 comprising the HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 21, and HCDR 3 of SEQ ID NO: 23; and

(ii) a nucleic acid sequence encoding the VL of SEQ ID NO: 27 comprising the LCDR1 of SEQ ID NO: 29, LCDR2 of SEQ ID NO: 31 and LCDR3 of SEQ ID NO: 33.

[00185] In another embodiment, the nucleic acid construct comprises:

(i) a nucleic acid sequence encoding the VH of SEQ ID NO: 1 comprising the HCDR1 of SEQ ID NO: 3, HCDR2 of SEQ ID NO: 5, and HCDR 3 of SEQ ID NO: 7; and

(ii) a nucleic acid sequence encoding the VL of SEQ ID NO: 43 comprising the LCDR1 of SEQ ID NO: 45, LCDR2 of SEQ ID NO: 47 and LCDR3 of SEQ ID NO: 33.

[00186] In another embodiment, the nucleic acid construct comprises:

(i) a nucleic acid sequence encoding the VH of SEQ ID NO: 1 comprising the HCDR1 of SEQ ID NO: 3, HCDR2 of SEQ ID NO: 5, and HCDR 3 of SEQ ID NO: 7; and

(ii) a nucleic acid sequence encoding the VL of SEQ ID NO: 52 comprising the LCDR1 of SEQ ID NO: 54, LCDR2 of SEQ ID NO: 56 and LCDR3 of SEQ ID NO: 58.

[00187] In some embodiments, the nucleic acid construct comprises a nucleic acid sequence encoding a scFv of one of SEQ ID NOs: 62, 64 and 66.

[00188] In some embodiments, the nucleic acid construct comprises a nucleic acid sequence encoding an anti-HLA-G CAR of SEQ ID NO: 68.

[00189] Also provided herein is a nucleic acid molecule comprising a nucleotide sequence encoding an anti-HLA-G CAR described herein and a nucleotide sequence encoding one, two or three costimulatory molecules. In some embodiments, the costimulatory molecule is one or both of CD40-L or 4-1BB-L.

[00190] Further provided herein is a nucleic acid molecule comprising a nucleotide sequence encoding an anti-HLA-G CAR described herein and a nucleotide sequence encoding one, two or three degrading enzymes.

[00191] The sequence of the open reading frame encoding the chimeric receptor can be obtained from a genomic DNA source, a cDNA source, generated by PCR from a cDNA source or else. Otherwise it can be chemically synthesized or combinations thereof. Depending upon the size of the genomic DNA and the number of introns, it may be desirable to use cDNA or a combination thereof as it is found that introns stabilize the mRNA or provide immune cell,

particularly T cell-specific expression (Barthel and Goldfeld, 2003). Also, it may be further advantageous to use endogenous or exogenous non-coding regions to stabilize the mRNA.

[00192] For expression of a chimeric antigen receptor of the present disclosure, the naturally occurring or endogenous transcriptional initiation region of the nucleic acid sequence encoding N-terminal components of the chimeric receptor can be used to generate the chimeric receptor in the target host cell. Alternatively, an exogenous transcriptional initiation region can be used that allows for constitutive or inducible expression, wherein expression can be controlled depending upon the target host, the level of expression desired, the nature of the target host, and the like.

[00193] Likewise, a signal sequence directing the chimeric receptor to the surface membrane can be the endogenous signal sequence of N-terminal component of the chimeric receptor. Optionally, in some instances, it may be desirable to exchange this sequence for a different signal sequence. However, the signal sequence selected should be compatible with the secretory pathway of the immune cell that will express the CAR so that the chimeric receptor is presented on the surface of the cell.

[00194] In accordance with the present disclosure, the nucleic acid construct is transformed or introduced into a cell and is transcribed and translated to produce a product (i.e. a chimeric receptor). Thus, the nucleic acid construct can further include at least one promoter for directing transcription of the CAR.

[00195] In one embodiment, the promoter is operably linked to the nucleic acid sequence encoding the chimeric receptor of the present disclosure, i.e., they are positioned so as to promote transcription of the messenger RNA from the DNA encoding the chimeric receptor. The promoter can be of genomic origin or synthetically generated. A variety of promoters for use in immune cells and particularly in T cells are well-known in the art (e.g., the CD4 promoter disclosed by Marodon et al. (2003)). The promoter can be constitutive or inducible, where induction is associated with the specific cell type or a specific level of maturation, or drug (e.g., tetracycline or doxorubicin) for example. Examples of inducible promoters include, but are not limited to, a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter. Alternatively, a number of well-known viral promoters are also suitable. Promoters of interest include the β-actin promoter, SV40 early and late promoters, immunoglobulin promoter, human cytomegalovirus promoter, retrovirus promoter, and the Friend spleen focus-forming virus promoter, mouse mammary tumor virus (MMTV), human

immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, avian leukemia virus promoter, Cytomegalovirus immediate early promoter, Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. The promoters may or may not be associated with enhancers, wherein the enhancers may be naturally associated with the particular promoter or associated with a different promoter.

[00196] Similarly, a termination region may be provided by the naturally occurring or endogenous transcriptional termination region of the nucleic acid sequence encoding the C-terminal component of the chimeric receptor. Alternatively, the termination region may be derived from a different source. For the most part, the source of the termination region is generally not considered to be critical to the expression of a recombinant protein and a wide variety of termination regions can be employed without adversely affecting expression. As will be appreciated by one skilled in the art that, in some instances, a few amino acids at the ends of the antigen binding domain in the CAR can be deleted, usually not more than 10, more usually not more than 5 residues, for example. Also, it may be desirable to introduce a small number of amino acids at the borders, usually not more than 10, more usually not more than 5 residues. The deletion or insertion of amino acids may be as a result of the needs of the construction, providing for convenient restriction sites, ease of manipulation, improvement in levels of expression, or the like. In addition, the substitute of one or more amino acids with a different amino acid can occur for similar reasons, usually not substituting more than about five amino acids in any one domain.

[00197] In another embodiment, the nucleic acid construct further comprises a promoter, the correct translation initiation sequence such as a ribosomal binding site and a start codon, a termination codon, and a transcription termination sequence.

[00198] The nucleic acid construct according to the disclosure may also comprise other regulatory regions such as enhancers, silencers and boundary elements/insulators to direct the level of transcription of a given gene.

[00199] The nucleic acid construct that encodes the chimeric receptor according to the disclosure can be prepared in conventional ways. Because, for the most part, natural sequences may be employed, the natural genes may be isolated and manipulated, as appropriate, so as to allow for the proper joining of the various components. Thus, the nucleic acid sequences encoding for the N-terminal and C-terminal proteins of the chimeric receptor can be isolated

by employing the polymerase chain reaction (PCR), using appropriate primers that result in deletion of the undesired portions of the gene. Alternatively, restriction digests of cloned genes can be used to generate the chimeric construct. In either case, the sequences can be selected to provide for restriction sites that are blunt-ended, or have complementary overlaps.

[00200] Polynucleotides can be prepared, manipulated and/or expressed using any of a variety of well-established techniques known and available in the art.

Vectors

[00201] In order to express a CAR described herein, an expression cassette encoding the CAR can be inserted into appropriate vector. The term "vector" is used herein to refer to a nucleic acid molecule capable transferring or transporting another nucleic acid molecule. The transferred nucleic acid is generally linked to, *e.g.*, inserted into, the vector nucleic acid molecule. A vector may include sequences that direct autonomous replication in a cell, or may include sequences sufficient to allow integration into host cell DNA.

[00202] The term "expression cassette" as used herein refers to genetic sequences within a vector which can express a RNA, and subsequently a protein. The nucleic acid cassette contains the gene of interest, *e.g.*, a CAR. The nucleic acid cassette is positionally and sequentially oriented within the vector such that the nucleic acid in the cassette can be transcribed into RNA, and when necessary, translated into a protein or a polypeptide, undergo appropriate post-translational modifications required for activity in the transformed cell, and be translocated to the appropriate compartment for biological activity by targeting to appropriate intracellular compartments or secretion into extracellular compartments. Preferably, the cassette has its 3' and 5' ends adapted for ready insertion into a vector, *e.g.*, it has restriction endonuclease sites at each end. In some embodiments, the nucleic acid cassette contains the sequence of a CAR used to increase the cytotoxicity of cancer cells that express HLA-G. The cassette can be removed and inserted into a plasmid or viral vector as a single unit.

[00203] Exemplary vectors include, without limitation, plasmids, phagemids, cosmids, transposons, artificial chromosomes such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC), bacteriophages such as lambda phage or M13 phage, and animal viruses. Examples of categories of animal viruses useful as vectors include, without limitation, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (*e.g.*, herpes simplex virus), poxvirus, baculovirus, papillomavirus, and papovavirus (*e.g.*, SV40). Examples of expression vectors are

pCIneo vectors (Promega) for expression in mammalian cells; pLenti4/V5-DEST™, pLenti6/V5-DEST™, and pLenti6.2/V5-GW/lacZ (Invitrogen) for lentivirus-mediated gene transfer and expression in mammalian cells. In some embodiments, the coding sequences of the CARs disclosed herein can be ligated into such expression vectors for the expression of the CARs in mammalian cells. In some embodiments, non-viral vectors are used to deliver one or more polynucleotides contemplated herein to a T cell.

[00204] In some embodiments, the vector is a non-integrating vector, including but not limited to, an episomal vector or a vector that is maintained extrachromosomally. As used herein, the term "episomal" refers to a vector that is able to replicate without integration into host's chromosomal DNA and without gradual loss from a dividing host cell also meaning that said vector replicates extrachromosomally or episomally. The vector is engineered to harbor the sequence coding for the origin of DNA replication or "ori" from a lymphotrophic herpes virus or a gamma herpesvirus, an adenovirus, SV40, a bovine papilloma virus, or a yeast, specifically a replication origin of a lymphotrophic herpes virus or a gamma herpesvirus corresponding to oriP of EBV. In a particular aspect, the lymphotrophic herpes virus may be Epstein Barr virus (EBV), Kaposi's sarcoma herpes virus (KSHV), Herpes virus saimiri (HS), or Marek's disease virus (MDV). Epstein Barr virus (EBV) and Kaposi's sarcoma herpes virus (KSHV) are also examples of a gamma herpesvirus. Typically, the host cell comprises the viral replication transactivator protein that activates the replication.

[00205] In some embodiments, a polynucleotide is introduced into a target or host cell using a transposon vector system. In certain embodiments, the transposon vector system comprises a vector comprising transposable elements and a polynucleotide contemplated herein; and a transposase. In one embodiment, the transposon vector system is a single transposase vector system, *see, e.g.,* WO 2008/027384. Exemplary transposases include, but are not limited to: piggyBac, Sleeping Beauty, Mos1, Tc1/mariner, Tol2, mini-Tol2, Tc3, MuA, Himar I, Frog Prince, and derivatives thereof. The piggyBac transposon and transposase are described, for example, in U.S. Patent 6,962,810, which is incorporated herein by reference in its entirety. The Sleeping Beauty transposon and transposase are described, for example, in Izsvak *et al.*, *J. Mol. Biol.* 302: 93-102 (2000), which is incorporated herein by reference in its entirety. The Tol2 transposon which was first isolated from the medaka fish *Oryzias latipes* and belongs to the hAT family of transposons is described in Kawakami *et al.* (2000). Mini-Tol2 is a variant of Tol2 and is described in Balciunas *et al.* (2006). The Tol2 and Mini-Tol2 transposons facilitate integration of a transgene into the genome of an organism when co-acting with the

Tol2 transposase. The Frog Prince transposon and transposase are described, for example, in Miskey *et al., Nucleic Acids Res.* 31:6873-6881 (2003).

[00206] Preferably, the vector according to the disclosure is a lentiviral vector. Particularly, the vector is derived from primate and non-primate lentivirus. Examples of primate lentiviruses include the human immunodeficiency virus (HIV), the causative agent of human acquired immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV). Prior art recombinant lentiviral vectors are known in the art, e.g., see US Patent Nos. 6,924,123; 7,056,699; 7,07,993; 7,419,829 and 7,442,551, incorporated herein by reference.

[00207] Commercial retroviral vectors for use in this disclosure include, but are not limited to, pFB-neo vectors (STRATAGENE®), Invitrogen's pLenti series versions 4, 6, and 6.2 "ViraPower" system. Manufactured by Lentigen Corp.; pHIV-7-GFP, lab generated and used by the City of Hope Research Institute; "Lenti-X" lentiviral vector, pLVX, manufactured by Clontech; pLKO.1-puro, manufactured by Sigma-Aldrich; pLemiR, manufactured by Open Biosystems; and pLV, lab generated and used by the Charité Medical School, Institute of Virology (CBF), Berlin, Germany.

[00208] It will be evident that a viral vector according to the disclosure need not be confined to the components of a particular virus. The viral vector may comprise components derived from two or more different viruses, and may also comprise synthetic components. Vector components can be manipulated to obtain desired characteristics, such as target cell specificity.

[00209] U.S. 6,924,123 discloses that certain retroviral sequence facilitate integration into the target cell genome. This patent teaches that each retroviral genome comprises genes called gag, pol and env which code for virion proteins and enzymes. These genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. They also serve as enhancer-promoter sequences. Accordingly, the vector according to the disclosure can comprise one or more of the integration features.

[00210] In some embodiment, the components of the particles not encoded by the vector genome are provided in trans by additional nucleic acid sequences (the "packaging system", which usually includes either or both of the gag/pol and env genes) expressed in the host cell,

for example using a helper virus strategy. The set of sequences required for the production of the viral vector particles may be introduced into the host cell by transient transfection, or they may be integrated into the host cell genome, or they may be provided in a mixture of ways, such as helper sequences. The techniques involved are known to those skilled in the art. For example, the retroviral constructs are packaging plasmids comprising at least one retroviral helper DNA sequence derived from a replication-incompetent retroviral genome encoding in trans all virion proteins required to package a replication incompetent retroviral vector, and for producing virion proteins capable of packaging the replication-incompetent retroviral vector at high titer, without the production of replication-competent helper virus.

[00211] In the packaging process, the packaging plasmids and retroviral vectors expressing the CAR according to the disclosure are transiently co-transfected into a first population of mammalian cells that are capable of producing virus, such as human embryonic kidney cells, for example 293 cells (ATCC No. CRL1573, ATCC, Rockville, Md.) to produce high titer recombinant retrovirus-containing supernatants. In another method of the disclosure this transiently transfected first population of cells is then co-cultivated with mammalian target cells, for example human lymphocytes, to transduce the target cells with the foreign gene at high efficiencies. In yet another method of the disclosure the supernatants from the above described transiently transfected first population of cells are incubated with mammalian target cells, for example human lymphocytes or hematopoietic stem cells, to transduce the target cells with the foreign gene at high efficiencies.

[00212] The nucleic acid construct according to the disclosure be inserted into a vector and packaged in retroviral or lentiviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either in vivo or ex vivo. In a particular aspect, the packaging plasmids are stably expressed in a first population of mammalian cells that are capable of producing virus, such as human embryonic kidney cells, for example 293 cells. Retroviral or lentiviral vectors are introduced into cells by either co-transfection with a selectable marker or infection with pseudotyped virus. In both cases, the vectors integrate. Alternatively, vectors can be introduced in an episomally maintained plasmid.

[00213] The "control elements" or "regulatory sequences" present in an expression vector are those non-translated regions of the vector (*e.g.*, origin of replication, selection cassettes, promoters, enhancers, translation initiation signals (Shine Dalgarno sequence or Kozak sequence) introns, a polyadenylation sequence, 5' and 3' untranslated regions) which interact

with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including ubiquitous promoters and inducible promoters may be used.

[00214] In some embodiments, vectors include, but are not limited to expression vectors and viral vectors, will include exogenous, endogenous, or heterologous control sequences such as promoters and/or enhancers. An "endogenous" control sequence is one which is naturally linked with a given gene in the genome. An "exogenous" control sequence is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques) such that transcription of that gene is directed by the linked enhancer/promoter. A "heterologous" control sequence is an exogenous sequence that is from a different species than the cell being genetically manipulated.

[00215] The term "promoter" as used herein refers to a recognition site of a polynucleotide (DNA or RNA) to which an RNA polymerase binds. An RNA polymerase initiates and transcribes polynucleotides operably linked to the promoter. In some embodiments, promoters operative in mammalian cells comprise an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated and/or another sequence found 70 to 80 bases upstream from the start of transcription, a CNCAAT region where N may be any nucleotide.

[00216] The term "enhancer" refers to a segment of DNA which contains sequences capable of providing enhanced transcription and in some instances can function independent of their orientation relative to another control sequence. An enhancer can function cooperatively or additively with promoters and/or other enhancer elements. The term "promoter/enhancer" refers to a segment of DNA which contains sequences capable of providing both promoter and enhancer functions.

[00217] The term "operably linked", refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. In one embodiment, the term refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, and/or enhancer) and a second polynucleotide sequence, *e.g.*, a polynucleotide-of-interest, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

[00218] Illustrative ubiquitous promoters suitable for use in some embodiments include, but are not limited to, a cytomegalovirus (CMV) immediate early promoter, a viral simian virus 40 (SV40) (*e.g.*, early or late) promoter, a spleen focus forming virus (SFFV) promoter, a Moloney murine leukemia virus (MoMLV) LTR promoter, a Rous sarcoma virus (RSV) LTR, a herpes simplex virus (HSV) (thymidine kinase) promoter, H5, P7.5, and P11 promoters from vaccinia virus, an elongation factor 1-alpha (EF1α) promoter, early growth response 1 (EGR1) promoter, a ferritin H (FerH) promoter, a ferritin L (FerL) promoter, a Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter, a eukaryotic translation initiation factor 4A1 (EIF4A1) promoter, a heat shock 70kDa protein 5 (HSPA5) promoter, a heat shock protein 90kDa beta, member 1 (HSP90B1) promoter, a heat shock protein 70kDa (HSP70) promoter, a β-kinesin (β-KIN) promoter, the human ROSA 26 locus (Irions *et al.*, *Nature Biotechnology* 25, 1477-1482 (2007)), a Ubiquitin C (UBC) promoter, a phosphoglycerate kinase-1 (PGK) promoter, a cytomegalovirus enhancer/chicken β-actin (CAG) promoter, a β-actin promoter and a myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted (MND) promoter (Challita *et al.*, *J Virol.* 69(2):748-55 (1995)).

[00219] In accordance with the present disclosure, the nucleic acid construct is transformed or introduced into a cell and is transcribed and translated to produce a product (i.e. a chimeric receptor). Thus, the nucleic acid construct can further include at least one promoter for directing transcription of the CAR.

[00220] In one embodiment, the promoter is operably linked to the nucleic acid sequence encoding the chimeric receptor of the present disclosure, i.e., they are positioned so as to promote transcription of the messenger RNA from the DNA encoding the chimeric receptor. The promoter can be of genomic origin or synthetically generated. A variety of promoters for use in immune cells and particularly in T cells are well-known in the art (e.g., the CD4 promoter disclosed by Marodon et al. (2003)). The promoter can be constitutive or inducible, where induction is associated with the specific cell type or a specific level of maturation, or drug (e.g., tetracycline or doxorubicin) for example. Examples of inducible promoters include, but are not limited to, a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter. Alternatively, a number of well-known viral promoters are also suitable. Promoters of interest include the β-actin promoter, SV40 early and late promoters, immunoglobulin promoter, human cytomegalovirus promoter, retrovirus promoter, and the Friend spleen focus-forming virus promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter,

avian leukemia virus promoter, Cytomegalovirus immediate early promoter, Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. The promoters may or may not be associated with enhancers, wherein the enhancers may be naturally associated with the particular promoter or associated with a different promoter.

[00221] In another embodiment, the nucleic acid construct further comprises a promoter, the correct translation initiation sequence such as a ribosomal binding site and a start codon, a termination codon, and a transcription termination sequence.

[00222] As used herein, "conditional expression" may refer to any type of conditional expression including, but not limited to, inducible expression; repressible expression; expression in cells or tissues having a particular physiological, biological, or disease state, *etc.* This definition is not intended to exclude cell type or tissue specific expression. Certain embodiments provide conditional expression of a polynucleotide-of-interest, *e.g.*, expression is controlled by subjecting a cell, tissue, organism, *etc.*, to a treatment or condition that causes the polynucleotide to be expressed or that causes an increase or decrease in expression of the polynucleotide encoded by the polynucleotide-of-interest. Illustrative examples of inducible promoters/systems include, but are not limited to, steroid-inducible promoters such as promoters for genes encoding glucocorticoid or estrogen receptors (inducible by treatment with the corresponding hormone), metallothionine promoter (inducible by treatment with various heavy metals), MX-1 promoter (inducible by interferon), the "GeneSwitch" mifepristone-regulatable system (Sirin *et al.*, 2003, *Gene*, 323:67), the cumate inducible gene switch (WO 2002/088346), tetracycline-dependent regulatory systems, *etc.*

[00223] Conditional expression can also be achieved by using a site-specific DNA recombinase. According to certain embodiments the vector comprises at least one (typically two) site(s) for recombination mediated by a site-specific recombinase. As used herein, the terms "recombinase" or "site specific recombinase" include excisive or integrative proteins, enzymes, co-factors or associated proteins that are involved in recombination reactions involving one or more recombination sites (*e.g.*, two, three, four, five, seven, ten, twelve, fifteen, twenty, thirty, fifty, *etc.*), which may be wild-type proteins (*see* Landy, *Current Opinion in Biotechnology* 3:699-707 (1993)), or mutants, derivatives (*e.g.*, fusion proteins containing the recombination protein sequences or fragments thereof), fragments, and variants thereof. Illustrative examples of recombinases suitable for use in particular embodiments

include, but are not limited to: Cre, Int, IHF, Xis, Flp, Fis, Hin, Gin, ΦC31, Cin, Tn3 resolvase, TndX, XerC, XerD, TnpX, Hjc, Gin, SpCCE1, and ParA.

[00224] As used herein, an "internal ribosome entry site" or "IRES" refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent translation of the gene. *See, e.g.*, Jackson *et al.*, 1990. *Trends Biochem Sci* 15(12):477-83) and Jackson and Kaminski. 1995. *RNA* 1(10):985-1000. In some embodiments, vectors include one or more polynucleotides-of-interest that encode one or more polypeptides. In some embodiments, to achieve efficient translation of each of the plurality of polypeptides, the polynucleotide sequences can be separated by one or more IRES sequences or polynucleotide sequences encoding self-cleaving polypeptides. In one embodiment, the IRES used in polynucleotides contemplated herein is an EMCV IRES.

[00225] Elements directing the efficient termination and polyadenylation of the heterologous nucleic acid transcripts increases heterologous gene expression. Transcription termination signals are generally found downstream of the polyadenylation signal. In some embodiments, vectors comprise a polyadenylation sequence 3′ of a polynucleotide encoding a polypeptide to be expressed. The term "polyA site" or "polyA sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript by RNA polymerase II. Polyadenylation sequences can promote mRNA stability by addition of a polyA tail to the 3′ end of the coding sequence and thus, contribute to increased translational efficiency. Cleavage and polyadenylation is directed by a poly(A) sequence in the RNA. The core poly(A) sequence for mammalian pre-mRNAs has two recognition elements flanking a cleavage-polyadenylation site. Typically, an almost invariant AAUAAA hexamer lies 20-50 nucleotides upstream of a more variable element rich in U or GU residues. Cleavage of the nascent transcript occurs between these two elements and is coupled to the addition of up to 250 adenosines to the 5' cleavage product. In some embodiments, the core poly(A) sequence is an ideal polyA sequence (e.g., AATAAA, ATTAAA, AGTAAA). In some embodiments, the poly(A) sequence is an SV40 polyA sequence, a bovine growth hormone polyA sequence (BGHpA), a rabbit β-globin polyA sequence (rβgpA), variants thereof, or another suitable heterologous or endogenous polyA sequence known in the art.

[00226] Illustrative methods of non-viral delivery of polynucleotides contemplated in some embodiments include, but are not limited to: electroporation, sonoporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, nanoparticles, polycation

or lipid:nucleic acid conjugates, naked DNA, artificial virions, DEAE-dextran-mediated transfer, gene gun, and heat-shock.

[00227] Illustrative examples of polynucleotide delivery systems suitable for use in some embodiments contemplated in some embodiments include, but are not limited to, those provided by Amaxa Biosystems, Maxcyte, Inc., BTX Molecular Delivery Systems, and Copernicus Therapeutics Inc. Lipofection reagents are sold commercially (*e.g.*, Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides have been described in the literature. See *e.g.*, Liu *et al.* (2003) *Gene Therapy.* 10:180–187; and Balazs *et al.* (2011) *Journal of Drug Delivery.* 2011:1-12. Antibody-targeted, bacterially derived, non-living nanocell-based delivery is also contemplated in some embodiments.

[00228] Viral vectors comprising polynucleotides contemplated in some embodiments can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (*e.g.*, intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (*e.g.*, mobilized peripheral blood, lymphocytes, bone marrow aspirates, tissue biopsy, *etc.*) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient.

[00229] In one embodiment, a viral vector comprising a polynucleotide encoding an anti-HLA-G CAR is administered directly to an organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells including, but not limited to, injection, infusion, topical application and electroporation. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

[00230] Illustrative examples of viral vector systems suitable for use in some embodiments contemplated herein include, but are not limited to adeno-associated virus (AAV), retrovirus, herpes simplex virus, adenovirus, and vaccinia virus vectors.

Genetically Modified Cells

[00231] In various embodiments, the present disclosure provides cells genetically modified to express the anti-HLA-G CARs described herein. As used herein, the term "genetically engineered" or "genetically modified" refers to the addition of extra genetic material in the form of DNA or RNA into the total genetic material in a cell. As used herein, the term "gene therapy" refers to the introduction of extra genetic material in the form of DNA or RNA into the total genetic material in a cell that restores, corrects, or modifies expression of a gene, or for the purpose of expressing a therapeutic polypeptide, *e.g.,* a CAR.

[00232] In some embodiments, the present disclosure provides genetically modified cells and populations thereof comprising an anti-HLA-G CAR. In some embodiments, the genetically modified cells comprise an anti-HLA-G CAR and one or more additional exogenous transgenes.

[00233] In some embodiments, the specificity of a primary immune effector cell is redirected to cells expressing HLA-G, *e.g.,* cancer cells, by genetically modifying the primary immune effector cell with a CAR contemplated herein. In various embodiments, a viral vector is used to genetically modify an immune effector cell with a particular polynucleotide encoding a CAR comprising an anti-HLA-G antigen binding domain that binds an HLA-G polypeptide; a hinge domain; a transmembrane (TM) domain, a short oligo- or polypeptide linker, that links the TM domain to the intracellular signaling domain of the CAR; and one or more intracellular co-stimulatory domains; and a signaling domain.

[00234] The cell can comprise a CAR that specifically binds to β2M-associated HLA-G isoforms, preferably to both HLA-G1 and HLA-G5 isoforms.

[00235] In one embodiment, the cell expresses at least two different CARs. For instance, the cell may comprise a CAR that specifically binds to β2M-associated HLA-G isoforms preferably to both HLA-G1 and HLA-G5, and another CAR that specifically binds to a different antigen.

[00236] Preferably, the cell comprises a CAR comprising an antigen binding fragment derived from the LFTT-1 antibody as described in WO2020043899, which is incorporated herein by reference in its entirety..

[00237] In some embodiments, a genetically modified cell comprises a CAR comprising the sequence of SEQ ID NO: 68 or a sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identity therewith.

[00238] The cell according to the disclosure can be a prokaryotic or a eukaryotic cell. Preferably, the cells are eukaryotic cells, such as mammalian cells, and typically are human, feline or canine cells, more typically human cells, preferably primary human cells.

Immune effector cells

[00239] In some embodiments, the present disclosure provides genetically modified cells (e.g., immune effector cells) and populations thereof comprising an anti-HLA-G CAR. In such embodiments, the anti-HLA-G CARs contemplated herein are introduced and expressed in immune effector cells so as to redirect the specificity of the immune cell to a target antigen of interest, *e.g.*, a HLA-G polypeptide. In some embodiments, the genetically modified cell expresses an anti-HLA-G CAR on the cell surface. In some embodiments, the genetically modified immune effector cells comprise an anti-HLA-G CAR and one or more additional exogenous transgenes.

[00240] An "immune effector cell," is any cell of the immune system that has one or more effector functions (*e.g.*, cytotoxic cell killing activity, secretion of cytokines, induction of ADCC and/or CDC). Exemplary immune effector cells include T lymphocytes, in particular cytotoxic T cells (CTLs; CD8+ T cells), TILs, and helper T cells (HTLs; CD4+ T cells), natural killer (NK) cells, and natural killer T (NKT) cells. Immune effector cells also include progenitors of effector cells wherein such progenitor cells can be induced to differentiate into immune effector cells *in vivo* or *in vitro*. In some embodiments, an immune effector cell is a Natural Killer (NK)-like cell, a hematopoietic progenitor cell, a peripheral blood (PB) derived T cell or an umbilical cord blood (UCB) derived T cell.

[00241] In some embodiments, anti-HLA-G CAR-modified immune effector cells comprise T cells. The terms "T cell" or "T lymphocyte" are art-recognized and are intended to include thymocytes, immature T lymphocytes, mature T lymphocytes, resting T lymphocytes, or activated T lymphocytes. A T cell can be a T helper (Th) cell, for example a T helper 1 (Th1) or a T helper 2 (Th2) cell. The T cell can be a helper T cell (HTL; $CD4^+$ T cell) $CD4^+$ T cell, a cytotoxic T cell (CTL; $CD8^+$ T cell), $CD4^+CD8^+$ T cell, $CD4^-CD8^-$ T cell, or any other subset of T cells. Other illustrative populations of T cells suitable for use in some embodiments include naïve T cells and memory T cells.

[00242] In some embodiments, the T cells are derived from a mammalian subject. In some embodiments, the T cells are derived from a primate subject, such as a human subject. T cells can be obtained from a number of sources including, but not limited to, peripheral blood

mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled person, such as sedimentation, *e.g.*, FICOLL™ separation. In one embodiment, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one embodiment, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing. The cells can be washed with PBS or with another suitable solution that lacks calcium, magnesium, and most, if not all other, divalent cations. As would be appreciated by those of ordinary skill in the art, a washing step may be accomplished by methods known to those in the art, such as by using a semiautomated flowthrough centrifuge. For example, the Cobe 2991 cell processor, the Baxter CytoMate, or the like. After washing, the cells may be resuspended in a variety of biocompatible buffers or other saline solution with or without buffer. In certain embodiments, the undesirable components of the apheresis sample may be removed in the cell directly resuspended culture media.

[00243] In certain embodiments, the immune cell is a T cell, e.g., an animal T cell, a mammalian T cell, a feline T cell, a canine T cell or a human T cell. Among the sub-types and subpopulations of T cells and/or of CD4+ and/or of CD8+ T cells are naive T (TN) cells, effector T cells (TEFF), memory T cells and sub-types thereof, such as stem cell memory T (TSCM), central memory T (TCM), effector memory T (TEM), or terminally differentiated effector memory T cells, tumor-infiltrating lymphocytes (TIL), immature T cells, mature T cells, helper T cells, cytotoxic T cells, mucosa-associated invariant T (MAIT) cells, naturally occurring and adaptive regulatory T (Treg) cells, helper T cells, such as TH1 cells, TH2 cells, TH3 cells, TH17 cells, TH9 cells, TH22 cells, follicular helper T cells, α/β T cells, and δ/γ T cells. Non-limiting examples of commercially available T-cell lines include lines BCL2 (AAA) Jurkat (ATCC® CRL-2902™), BCL2 (S70A) Jurkat (ATCC® CRL-2900™), BCL2 (S87A) Jurkat (ATCC® CRL-2901™), BCL2 Jurkat (ATCC® CRL-2899™), Neo Jurkat (ATCC® CRL-2898™), TALL-104 cytotoxic human T cell line (ATCC # CRL-11386). Further examples include but are not limited to mature T-cell lines, e.g., such as Deglis, EBT-8, HPB-MLp-W, HUT 78, HUT 102, Karpas 384, Ki 225, My-La, Se-Ax, SKW-3, SMZ-1 and T34; and immature T- cell lines, e.g., ALL-SIL, Be13, CCRF-CEM, CML-T1, DND-41, DU.528,

EU-9, HD-Mar, HPB-ALL, H-SB2, HT-1, JK-T1, Jurkat, Karpas 45, KE-37, KOPT-K1, K-T1, L-KAW, Loucy, MAT, MOLT-1, MOLT 3, MOLT-4, MOLT 13, MOLT-16, MT-1, MT-ALL, P12/Ichikawa, Peer, PER0117, PER-255, PF-382, PFI-285, RPMI-8402, ST-4, SUP-T1 to T14, TALL-1, TALL-101, TALL-103/2, TALL-104, TALL-105, TALL-106, TALL-107, TALL-197, TK-6, TLBR-1, -2, -3, and -4, CCRF-HSB-2 (CCL-120.1), J.RT3-T3.5 (ATCC TIB-153), J45.01 (ATCC CRL-1990), J.CaM1.6 (ATCC CRL-2063), RS4;11 (ATCC CRL-1873), CCRF-CEM (ATCC CRM-CCL-119); and cutaneous T-cell lymphoma lines, e.g., HuT78 (ATCC CRM-TIB-161), MJ[G11] (ATCC CRL-8294), HuT102 (ATCC TIB-162). Non-limiting exemplary sources for such commercially available cell lines include the American Type Culture Collection (ATCC) (Manassas, VA), and the German Collection of Microorganisms and Cell Cultures.

[00244] In some embodiments, a population of cells comprising T cells, *e.g.,* peripheral blood mononuclear cells (PBMCs), is genetically modified according to the present disclosure. In some embodiments, the population of PBMCs is not subjected to positive or negative selection prior to activation, expansion, and/or genetic modification. In other embodiments, T cells are isolated or purified from PBMCs prior to activation, expansion, and/or genetic modification. In such embodiments, the population of PBMCs can be treated to lyse the red blood cells and deplete the monocytes, for example, by centrifugation through a PERCOLL™ gradient. In some embodiments, cytotoxic and/or helper T lymphocytes are isolated from PBMCs. In some embodiments, the isolated T cells can be sorted into naïve, memory, and effector T cell subpopulations either before or after activation, expansion, and/or genetic modification. In certain embodiments, specific subpopulation of T cells, expressing one or more of the following markers: CD3, CD4, CD8, CD28, CD45RA, CD45RO, CD62, CD127, and HLA-DR can be further isolated by positive or negative selection techniques.

[00245] In some embodiments, immune effector cells include progenitors of immune effectors cells such as hematopoietic stem cells (HSCs) contained within the $CD34^+$ population of cells derived from cord blood, bone marrow, or mobilized peripheral blood, and which differentiate into mature immune effector cells upon administration in a subject, or which can be induced *in vitro* to differentiate into mature immune effector cells.

[00246] In some embodiments, the cells are natural killer (NK) cells, Natural Killer T (NKT) cells, cytokine-induced killer (CIK) cells, tumor-infiltrating lymphocytes (TILs), lymphokine- activated killer (LAK) cells, or the like. NK cells may either be isolated or obtained from a commercially available source. Non-limiting examples of commercial NK

cell lines include lines NK-92 (ATCC® CRL-2407™), NK-92MI (ATCC® CRL-2408™). Further examples include but are not limited to NK lines HANK1, KHYG-1, NKL, NK-YS, NOI-90, and YT. Non-limiting exemplary sources for such commercially available cell lines include the American Type Culture Collection (ATCC) (Manassas, VA) and the German Collection of Microorganisms and Cell Cultures.

[00247] In some embodiments, the host cell presenting the CAR according to the disclosure is selected from cytotoxic T cells (also known as TC, Cytotoxic T Lymphocyte, CTL, T Killer cell, a lytic T cell, CD8+ T cells or killer T cell) and NK cells.

[00248] In some embodiments, the cells are B cells, monocytes or granulocytes, e.g., myeloid cells, macrophages, neutrophils, dendritic cells, mast cells, eosinophils, and/or basophils.

[00249] In some embodiments, a cell expresses an anti-HLA-G CAR disclosed herein and additionally expresses one, two or three inhibitors of an immune checkpoint molecule. In some cases, the immune checkpoint molecule is PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM-1, CEACAM-3, CEACAM-5, LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GALS, adenosine or TGFR.

Genetic Modifications

[00250] In some embodiments, the genetically modified cells described herein comprise an anti-HLA-G CAR and further comprise one or more additional exogenous transgenes. In some embodiments, the one or more additional exogenous transgenes encode a detectable tag, a safety-switch system, or a chimeric switch receptor.

Detectable Tags

[00251] In some embodiments, the genetically modified cells described herein comprise an anti-HLA-G CAR and further comprise an exogenous transgene encoding a detectable tag. Examples of detectable tags include but are not limited to, FLAG tags, poly-histidine tags (*e.g.* 6xHis), SNAP tags, Halo tags, cMyc tags, glutathione-S-transferase tags, avidin, enzymes, fluorescent proteins, luminescent proteins, chemiluminescent proteins, bioluminescent proteins, and phosphorescent proteins.

[00252] In some embodiments the fluorescent protein is selected from the group consisting of blue/UV proteins (such as BFP, TagBFP, mTagBFP2, Azurite, EBFP2, mKalama1, Sirius, Sapphire, and T-Sapphire); cyan proteins (such as CFP, eCFP, Cerulean, SCFP3A,

mTurquoise, mTurquoise2, monomeric Midoriishi-Cyan, TagCFP, and mTFP1); green proteins (such as: GFP, eGFP, meGFP (A208K mutation), Emerald, Superfolder GFP, Monomeric Azami Green, TagGFP2, mUKG, mWasabi, Clover, and mNeonGreen); yellow proteins (such as YFP, eYFP, Citrine, Venus, SYFP2, and TagYFP); orange proteins (such as Monomeric Kusabira-Orange, mKOκ, mKO2, mOrange, and mOrange2); red proteins (such as RFP, mRaspberry, mCherry, mStrawberry, mTangerine, tdTomato, TagRFP, TagRFP-T, mApple, mRuby, and mRuby2); far-red proteins (such as mPlum, HcRed-Tandem, mKate2, mNeptune, and NirFP); near-infrared proteins (such as TagRFP657, IFP1.4, and iRFP); long stokes shift proteins (such as mKeima Red, LSS-mKate1, LSS-mKate2, and mBeRFP); photoactivatible proteins (such as PA-GFP, PAmCherry1, and PATagRFP); photoconvertible proteins (such as Kaede (green), Kaede (red), KikGR1 (green), KikGR1 (red), PS-CFP2, PS-CFP2, mEos2 (green), mEos2 (red), mEos3.2 (green), mEos3.2 (red), PSmOrange, and PSmOrange); and photoswitchable proteins (such as Dronpa). In some embodiments, the detectable tag can be selected from AmCyan, AsRed, DsRed2, DsRed Express, E2-Crimson, HcRed, ZsGreen, ZsYellow, mCherry, mStrawberry, mOrange, mBanana, mPlum, mRaspberry, tdTomato, DsRed Monomer, and/or AcGFP, all of which are available from Clontech.

[00253] In some embodiments, the detectable tag and the anti-HLA-G CAR are expressed from the same expression cassette. For example, in some embodiments, the genetically modified cells described herein comprise an expression cassette comprising a first polynucleotide sequence encoding an anti-HLA-G CAR and a second polynucleotide sequence encoding a detectable tag.

Manufacturing Methods

[00254] Methods of introducing genes into a cell and expressing genes in a cell are known in the art.

[00255] Particularly, methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means that are more particularly described here below.

[00256] In some embodiments, the methods include isolating cells from the subject, preparing, processing, culturing, and/or engineering them, as described herein, and re-introducing them into the same patient, before or after cryopreservation.

[00257] Here is particularly provided a method of producing anti-HLA-G CAR expressing cells comprising, or alternatively consisting essentially of, or yet further consisting of the steps: (i) transducing a population of isolated cells with a nucleic acid sequence encoding the CAR as described herein; and (ii) selecting a subpopulation of said isolated cells that have been successfully transduced with said nucleic acid sequence of step (i) thereby producing anti-HLA-G CAR expressing cells. In one aspect, the isolated cells are selected from a group consisting of T cells and NK cells.

[00258] Here is even more particularly provided a method of producing anti-HLA-G CAR expressing cells comprising, or alternatively consisting essentially of, or yet further consisting of the steps: (i) acquisition of an immune cell population (e.g. blood cells) (ii) isolation of a particular cell population (e.g. T cells and/or NK cells) (iii) transducing a population of isolated cells with a nucleic acid sequence encoding the CAR as described herein; and (iv) selecting a subpopulation of said isolated cells that have been successfully transduced with said nucleic acid sequence of step (iii) thereby producing anti HLA-G CAR expressing cells. These different steps are more particularly described below.

Cell acquisition

[00259] Prior to expansion and genetic modification of the cells disclosed herein, cells may be obtained from a subject – for instance, in embodiments involving autologous therapy – or from a commercially available culture.

[00260] The cell can be acquired from samples include tissue, body fluid (e.g. blood, plasma, serum, cerebrospinal fluid, synovial fluid, urine and sweat), and other samples taken directly from the subject, as well as samples resulting from one or more processing steps, such as separation, centrifugation, genetic engineering (e.g. transduction with viral vector), washing, and/or incubation.

[00261] Cells can be obtained from a number of non-limiting sources including whole blood, peripheral blood mononuclear cells (PBMCs), leukocytes, bone marrow, thymus tissue, lymph node tissue, cord blood, tissue from a site of infection, ascites, pleural effusion, tissue biopsy, tumor, leukemia, lymphoma, gut associated lymphoid tissue, mucosa associated lymphoid tissue, spleen, other lymphoid tissues, liver, lung, stomach, intestine, colon, kidney, pancreas,

breast, bone, prostate, cervix, testes, ovaries, tonsil, or other organ, and/or cells derived therefrom. Samples include, in the context of cell therapy, e.g. adoptive cell therapy, samples from autologous and allogeneic sources.

[00262] In some examples, cells from the circulating blood of a subject are obtained, e.g., by apheresis or leukapheresis. The samples, in some aspects, contain lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and/or platelets, and in some aspects contain cells other than red blood cells and platelets.

[00263] In some embodiments, any number of T cell ok NK cell lines available and known to those skilled in the art, such as described hereabove may be used. In some embodiments, cells can be derived from a healthy donor, from a patient diagnosed with cancer, from a patient diagnosed with an autoimmune or inflammatory disorder or from a patient diagnosed with an infection. In some embodiments, cells can be part of a mixed population of cells which present different phenotypic characteristics.

Cell isolation

[00264] As is known to one of skill in the art, various methods are readily available for isolating immune cells from a subject or can be adapted to the present application, for example using Life Technologies Dynabeads® system; STEMcell Technologies EasySep™, RoboSep™, RosetteSep™, SepMate™; Miltenyi Biotec MACS™ cell separation kits, cell surface marker expression and other commercially available cell separation and isolation kits (e.g., ISOCELL from Pierce, Rockford, IL). Particular subpopulations of immune cells may be isolated through the use of beads or other binding agents available in such kits specific to unique cell surface markers. For example, MACS™ CD4+ and CD8+ MicroBeads may be used to isolate CD4+ and CD8+ T-cells. The strategy of isolating and expanding antigen-specific T cells as a therapeutic intervention for human disease has also been validated in clinical trials (Riddell et al., 1992; Walter et al., 1995; Heslop et al., 1996).

[00265] In some embodiments, isolation of the cells includes one or more preparation and/or non-affinity-based cell separation steps. In some examples, cells are washed, centrifuged, and/or incubated in the presence of one or more reagents, for example, to remove unwanted components, enrich for desired components, lyse or remove cells sensitive to particular reagents. In some examples, cells are separated based on one or more property, such as density, adherent properties, size, sensitivity and/or resistance to particular components.

[00266] In some embodiments, the blood cells collected from the subject are washed, e.g., to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In some embodiments, the cells are washed with phosphate buffered saline (PBS). In some embodiments, the wash solution lacks calcium and/or magnesium and/or many or all divalent cations. In some aspects, a washing step is accomplished by a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor, Baxter) according to the manufacturer's instructions. In some aspects, a washing step is accomplished by tangential flow filtration (TFF) according to the manufacturer's instructions. In some embodiments, the cells are resuspended in a variety of biocompatible buffers after washing, such as, for example, Ca2+/Mg2+ free PBS. In certain embodiments, components of a blood cell sample are removed and the cells directly resuspended in culture media.

[00267] In some embodiments, the isolation methods include the separation of different cell types based on the expression or presence in the cell of one or more specific molecules, such as surface markers, e.g., surface proteins, intracellular markers or nucleic acid. In some embodiments, any known method for separation based on such markers may be used. In some embodiments, the separation is affinity- or immunoaffinity-based separation. For example, the isolation in some aspects includes separation of cells and cell populations based on the cells' expression or expression level of one or more markers, typically cell surface markers, for example, by incubation with an antibody or binding partner that specifically binds to such markers, followed generally by washing steps and separation of cells having bound the antibody or binding partner, from those cells having not bound to the antibody or binding partner. Such separation steps can be based on positive selection, in which the cells having bound the reagents are retained for further use, and/or negative selection, in which the cells having not bound to the antibody or binding partner are retained. In some examples, both fractions are retained for further use. In some aspects, negative selection can be particularly useful where no antibody is available that specifically identifies a cell type in a heterogeneous population, such that separation is best carried out based on markers expressed by cells other than the desired population.

[00268] In some embodiments, multiple rounds of separation steps are carried out, where the positively or negatively selected fraction from one step is subjected to another separation step, such as a subsequent positive or negative selection. In some examples, a single separation step can deplete cells expressing multiple markers simultaneously, such as by incubating cells with

a plurality of antibodies or binding partners, each specific for a marker targeted for negative selection. Likewise, multiple cell types can simultaneously be positively selected by incubating cells with a plurality of antibodies or binding partners expressed on the various cell types.

[00269] For example, in some aspects, specific subpopulations of T cells, such as cells positive or expressing high levels of one or more surface markers, e.g., CD28+, CD62L+, CCR7+, CD27+, CD127+, CD4+, CD8+, CD45RA+, and/or CD45RO+ T cells, are isolated by positive or negative selection techniques. For example, CD3+ T cells can be expanded using CD3 / CD28 conjugated magnetic beads (e.g., DYNABEADS® M-450 CD3/CD28 T Cell Expander).

[00270] In some embodiments, isolation is carried out by enrichment for a particular cell population by positive selection, or depletion of a particular cell population, by negative selection. In some embodiments, positive or negative selection is accomplished by incubating cells with one or more antibodies or other binding agent that specifically bind to one or more surface markers expressed or expressed (marker+) at a relatively higher level on the positively or negatively selected cells, respectively.

[00271] In some embodiments, T cells are separated from a sample by negative selection of markers expressed on non-T cells, such as B cells, monocytes, or other white blood cells, such as CD14. In some aspects, a CD4 or CD8 selection step is used to separate CD4+ helper and CD8+ cytotoxic T cells. Such CD4+ and CD8+ populations can be further sorted into sub-populations by positive or negative selection for markers expressed or expressed to a relatively higher degree on one or more naive, memory, and/or effector T cell subpopulations.

[00272] In some embodiments, CD8+ cells are further enriched for or depleted of naive, central memory, effector memory, and/or central memory stem cells, such as by positive or negative selection based on surface antigens associated with the respective subpopulation. In some embodiments, enrichment for central memory T (TCM) cells is carried out to increase efficacy, such as to improve long-term survival, expansion, and/or engraftment following administration, which in some aspects is particularly robust in such sub-populations. See Terakura et al. (2012) Blood. 1:72-82; Wang et al. (2012) J Immunother. 35(9):689-701. In some embodiments, combining TCM-enriched CD8+ T cells and CD4+ T cells further enhances efficacy.

[00273] In embodiments, memory T cells are present in both CD62L+ and CD62L- subsets of CD8+ peripheral blood lymphocytes. PBMC can be enriched for or depleted of CD62L- CD8+ and/or CD62L+CD8 fractions, such as using anti-CD8 and anti-CD62L antibodies.

[00274] In some embodiments, the enrichment for central memory T (TCM) cells is based on positive or high surface expression of CD45RO, CD62L, CCR7, CD28, CD3, and/or CD127; in some aspects, it is based on negative selection for cells expressing or highly expressing CD45RA and/or granzyme B. In some aspects, isolation of a CD8+ population enriched for TCM cells is carried out by depletion of cells expressing CD4, CD14, CD45RA, and positive selection or enrichment for cells expressing CD62L. In one aspect, enrichment for central memory T (TCM) cells is carried out starting with a negative fraction of cells selected based on CD4 expression, which is subjected to a negative selection based on expression of CD14 and CD45RA, and a positive selection based on CD62L. Such selections in some aspects are carried out simultaneously and in other aspects are carried out sequentially, in either order. In some aspects, the same CD4 expression-based selection step used in preparing the CD8+ cell population or subpopulation, also is used to generate the CD4+ cell population or sub-population, such that both the positive and negative fractions from the CD4 based separation are retained and used in subsequent steps of the methods, optionally following one or more further positive or negative selection steps.

[00275] In some embodiments, the enrichment for NK cells is based on positive or high surface expression of CD56 and CD16 and on the negative expression of CD3 and/or optionally on the presence of NKp46 or NKp30 receptors.

[00276] In some aspects, the sample or composition of cells to be separated is incubated with small, magnetizable or magnetically responsive material, such as magnetically responsive particles or micro-particles, such as paramagnetic beads (e.g., such as Dynabeads or MACS beads). The magnetically responsive material, e.g., particle, generally is directly or indirectly attached to a binding partner, e.g., an antibody, that specifically binds to a molecule, e.g., surface marker, present on the cell, cells, or population of cells that it is desired to separate, e.g., that it is desired to negatively or positively select. In some embodiments, the magnetic particle or bead comprises a magnetically responsive material bound to a specific binding member, such as an antibody or other binding partner. There are many well-known magnetically responsive materials used in magnetic separation methods. Suitable magnetic particles include those described in Molday, U. S. Pat. No. 4,452,773, and in European Patent Specification EP 452342 B, which are hereby incorporated by reference. Colloidal sized particles, such as those described in Owen U. S. Pat. No. 4,795,698, and Liberti et al., U. S. Pat. No. 5,200,084 are other examples.

[00277] The incubation generally is carried out under conditions whereby the antibodies or binding partners or molecules, such as secondary antibodies or other reagents, which specifically bind to such antibodies or binding partners, which are attached to the magnetic particle or bead, specifically bind to cell surface molecules if present on cells within the sample.

[00278] In some aspects, the sample is placed in a magnetic field, and those cells having magnetically responsive or magnetizable particles attached thereto will be attracted to the magnet and separated from the unlabeled cells. For positive selection, cells that are attracted to the magnet are retained; for negative selection, cells that are not attracted (unlabeled cells) are retained. In some aspects, a combination of positive and negative selection is performed during the same selection step, where the positive and negative fractions are retained and further processed or subject to further separation steps.

[00279] In certain embodiments, the magnetically responsive particles are coated in primary antibodies or other binding partners, secondary antibodies, lectins, enzymes, or streptavidin. In certain embodiments, the magnetic particles are attached to cells via a coating of primary antibodies specific for one or more markers. In certain embodiments, the cells, rather than the beads, are labeled with a primary antibody or binding partner, and then cell-type specific secondary antibody- or other binding partner (e.g., streptavidin)-coated magnetic particles, are added. In certain embodiments, streptavidin-coated magnetic particles are used in conjunction with biotinylated primary or secondary antibodies.

[00280] In some embodiments, the magnetically responsive particles are left attached to the cells that are to be subsequently incubated, cultured and/or engineered; in some aspects, the particles are left attached to the cells for administration to a patient. In some embodiments, the magnetizable or magnetically responsive particles are removed from the cells. Methods for removing magnetizable particles from cells are known and include, e.g., the use of competing non-labeled antibodies, magnetizable particles or antibodies conjugated to cleavable linkers, etc. In some embodiments, the magnetizable particles are biodegradable.

[00281] In certain embodiments, the isolation or separation is carried out using a system, device, or apparatus that carries out one or more of the isolation, cell preparation, separation, processing, incubation, culture, and/or formulation steps of the methods. In some aspects, the system is used to carry out each of these steps in a closed or sterile environment, for example, to minimize error, user handling and/or contamination. In one example, the system is a system

as described in International Patent Application, Publication Number WO2009/072003, or US 20110003380.

[00282] In some embodiments, a cell population described herein is collected and enriched (or depleted) via flow cytometry, in which cells stained for multiple cell surface markers are carried in a fluidic stream. In some embodiments, a cell population described herein is collected and enriched (or depleted) via preparative scale (FACS)-sorting. In certain embodiments, a cell population described herein is collected and enriched (or depleted) by use of microelectromechanical systems (MEMS) chips in combination with a FACS-based detection system (see, e.g., WO 2010/033140, Cho et al. (2010) Lab Chip 10, 1567-1573; and Godin et al. (2008) J Biophoton. l(5):355-376. In both cases, cells can be labeled with multiple markers, allowing for the isolation of well-defined T cell subsets at high purity. In some embodiments, the antibodies or binding partners are labeled with one or more detectable marker, to facilitate separation for positive and/or negative selection. For example, separation may be based on binding to fluorescently labeled antibodies. In some examples, separation of cells based on binding of antibodies or other binding partners specific for one or more cell surface markers are carried in a fluidic stream, such as by fluorescence-activated cell sorting (FACS), including preparative scale (FACS) and/or microelectromechanical systems (MEMS) chips, e.g., in combination with a flow-cytometric detection system. Such methods allow for positive and negative selection based on multiple markers simultaneously.

[00283] In some embodiments, the methods include density-based cell separation methods, such as the preparation of white blood cells from peripheral blood by lysing the red blood cells and centrifugation through a Percoll or Ficoll gradient.

[00284] In any of the aforementioned separation steps, the separation does not result in 100% enrichment or removal of a particular cell population or cells expressing a particular marker. For example, positive selection of or enrichment for cells of a particular type, such as those expressing a marker, refers to increasing the number or percentage of such cells, but does not result in a complete absence of cells not expressing the marker. Likewise, negative selection, removal, or depletion of cells of a particular type, such as those expressing a marker, refers to decreasing the number or percentage of such cells, but does not result in a complete removal of all such cells.

[00285] Alternatively, cells may be obtained through commercially available cell cultures, including but not limited to, for T cells, lines BCL2 (AAA) Jurkat (ATCC® CRL-2902™),

BCL2 (S70A) Jurkat (ATCC® CRL-2900™), BCL2 (S87A) Jurkat (ATCC® CRL-2901™), BCL2 Jurkat (ATCC® CRL-2899™), Neo Jurkat (ATCC® CRL-2898™), TALL-104 (ATTC® CRL-11386); and, for NK cells, lines NK-92 (ATCC® CRL-2407™), NK-92MI (ATCC® CRL-2408™).

[00286] Non-limiting exemplary sources for such commercially available cell lines include the American Type Culture Collection (ATCC) (Manassas, VA) and the German Collection of Microorganisms and Cell Cultures.

Cell preparation and expansion

[00287] Whether prior to or after genetic modification of the immune cells to express a desirable CAR, the cells can be activated and expanded using generally known methods or from readily adapted method to the present application such as those described in U.S. Patent Nos.6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7, 172,869; 7,232,566; 7, 175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041 and U.S. Patent Application Publication No. 20060121005, Life Technologies Dynabeads® system activation and expansion kits; BD Biosciences Phosflow™ activation kits, Miltenyi Biotec MACS™ activation/expansion kits, and other commercially available cell kits specific to activation moieties of the relevant cell. Stimulation with the HLA-G antigen ex vivo can activate and expand the selected CAR expressing cell subpopulation. Alternatively, the cells may be activated in vivo by interaction with HLA-G antigen.

[00288] The incubation and/or engineering may be carried out in a culture vessel, such as a unit, chamber, well, column, tube, tubing set, valve, vial, culture dish, bag, or other container for culture or cultivating cells.

[00289] In some embodiments, the cells are incubated and/or cultured prior to or in connection with genetic engineering. The incubation steps can include culture, cultivation, stimulation, activation, and/or propagation. In some embodiments, the compositions or cells are incubated in the presence of stimulating conditions or a stimulatory agent. Such conditions include those designed to induce proliferation, expansion, activation, and/or survival of cells in the population, to mimic antigen exposure, and/or to prime the cells for genetic engineering, such as for the introduction of a recombinant antigen receptor. The conditions can include one or more of particular media, temperature, oxygen content, carbon dioxide content, time, agents, e.g., nutrients, amino acids, antibiotics, ions, and/or stimulatory factors, such as cytokines,

chemokines, antigens, binding partners, fusion proteins, recombinant soluble receptors and any other agents designed to activate the cells.

[00290] In some embodiments, the immune cells of the disclosure can be expanded in vitro by co- culturing with tissue or cells. The cells can also be expanded in vivo, for example in the subject's blood after administrating the cell into the subject.

[00291] Generally, the T cells of the disclosure can be expanded, for example, by contact with an agent that stimulates a CD3 TCR complex and a co-stimulatory molecule on the surface of the T cells to create an activation signal for the T cell. For example, chemicals such as calcium ionophore A23187, phorbol 12-myristate 13- acetate (PMA), or mitogenic lectins like phytohemagglutinin (PHA) can be used to create an activation signal for the T cell.

[00292] In some embodiments, T cell populations may be stimulated in vitro by contact with, for example, an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore. In some embodiments, the T cell populations may be stimulated in vitro by contact with Muromonab-CD3 (OKT3). For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be incubated with an anti-CD3 antibody and an anti-CD28 antibody under conditions stimulating proliferation of the T cells.

[00293] In some embodiments, the T cells are expanded by adding to the culture-initiating composition feeder cells, such as non-dividing PBMC, (e.g., such that the resulting population of cells contains at least about 5, 10, 20, or 40 or more PBMC feeder cells for each T lymphocyte in the initial population to be expanded); and incubating the culture (e.g. for a time sufficient to expand the numbers of T cells). In some aspects, the non-dividing feeder cells can comprise gamma-irradiated PBMC feeder cells. In some embodiments, the PBMC are irradiated with gamma rays in the range of about 3000 to 3600 rads to prevent cell division. In some aspects, the feeder cells are added to culture medium prior to the addition of the populations of T cells.

[00294] In some embodiments, co-stimulatory molecules are employed to enhance the activation, proliferation, and cytotoxicity of T cells produced by the CAR after antigen engagement. A co-stimulatory ligand can include, but is not limited to, B7-1 (CD80), B7-2 (CD86), B7-H3, BAFFR, BTLA, BLAME (SLAMF8), CD2, CD4, CD5, CD7, CD8a, CD8β,

CD1a, LFA-1 (CD11a/CD18), CD1b, CD1c, CD1d, CD18, CD19, CD19a, CD27, CD28, CD29, CD30, CD30L, CD40, CD40MICA, CD49a, CD49D, CD49f, CD69, , CD70, CD83, CD84, CD96 (Tactile), CD 100 (SEMA4D), CD 103, OX40 (CD134), 4-1BB (CD137), SLAM (SLAMF1, CD150, IPO-3), CD160 (BY55), SELPLG (CD 162), DNAM1 (CD226), Ly9 (CD229), SLAMF4 (CD244, 2B4), ICOS (CD278), CEACAM1, CDS, CRTAM, DAP10, GADS, GITR, HVEM (LIGHTR), HLA-G, IA4, ICAM-1, IL2R β, IL2R γ, IL7R a, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB1, ITGB2, ITGB7, KIRDS2, LAT, LFA-1, LIGHT, LTBR, MICB, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), PAG/Cbp, PD-1, PD-L1, PD-L2, PSGL1, SLAMF6 (NTB-A, Ly108), SLAMF7, SLP-76, TNFR2, TRANCE/RANKL, VLA1, VLA-6, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (rCAM), lymphotoxin beta receptor,3 TR6, ILT3 and ILT4. A co-stimulatory ligand also encompasses, inter alia, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as, but not limited to, CD27, CD28, 4- 1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen- 1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83.

[00295] In some embodiments, NK cell populations can be expanded in vitro using interleukin-2 (IL-2) IL-15, IL-15/IL-15RA complex, IL-18 and IL-12.

[00296] Conditions appropriate for T cell and NK cell culture include an appropriate media (e.g., Minimal Essential Media or RPMI Media 1640 or, X-Vivo 10, X-Vivo 15 and X-Vivo 20 (Lonza)) that may contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-γ, IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-2, IL-15, IL-18, IL-21, TGF, and TNF, or any other additives for the growth of cells known to the skilled artisan. In a preferred embodiment, T cells are stimulated in vitro by exposure to OKT3 and IL-2. Other additives for the growth of cells include, but are not limited to, surfactant, Plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, AIM-V, DMEM, MEM, a-MEM, F-12, X-Vivo 10, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells.

[00297] Antibiotics, e.g., penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate

temperature (e.g., 37 degrees Celsius) and atmosphere (e.g., air plus 5% CO2). T cells that have been exposed to varied stimulation times may exhibit different characteristics.

[00298] In some embodiments, the preparation methods include steps for freezing, e.g., cryopreserving, the cells, either before or after isolation, incubation, and/or engineering. In some embodiments, the freeze and subsequent thaw step removes granulocytes and, to some extent, monocytes in the cell population. In some embodiments, the cells are suspended in a freezing solution, e.g., following a washing step to remove plasma and platelets. Any of a variety of known freezing solutions and parameters in some aspects may be used. One example involves using PBS containing 20% DMSO and 8% human serum albumin (HSA), or other suitable cell freezing media. This is then diluted 1:1 with media so that the final concentration of DMSO and HSA are 10% and 4%, respectively. The cells are then frozen to -80°C at a rate of 1 degree per minute and stored in the vapor phase of a liquid nitrogen storage tank.

[00299] In some embodiments, the NK cells or T cells are *ex vivo* expanded for at least about 5 days, for example, not less than about 10 days, not less than about 15 days, or not less than about 20 days before administration to the patient.

[00300] In some embodiments, the NK cells or T cells have been expanded at least about 100-fold, preferably at least about 200-fold, and more preferably at least about 400-fold, preferably at least about 600-fold, more preferably at least about 1000 fold and even more preferably at least about 1500 fold compared to day 0 of expansion, before administration to a patient.

Cell transduction and expansion

[00301] The nucleic acid construct according to the disclosure can be transduced into immune cells to create an immune cell that expresses the anti-HLA-G CAR according to the disclosure. In certain embodiments, cells are transduced to comprise at least one CAR of the present disclosure.

[00302] It is contemplated that the chimeric nucleic acid construct can be introduced into the subject's own immune cells as naked DNA or in a suitable vector. Methods of stably transfecting immune cells, particularly T cell, by electroporation using naked DNA are known in the art. See e.g., U.S. Pat. No. 6,410,319. Naked DNA generally refers to the DNA encoding a chimeric receptor of the present disclosure contained in a plasmid expression vector in proper orientation for expression. Advantageously, the use of naked DNA reduces the time required to produce T cells expressing the chimeric receptor of the present disclosure. Physical methods

for introducing a nucleic acid construct into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like.

[00303] Alternatively, biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. A variety of viral vectors such as vector described hereabove can be used to introduce the nucleic acid construct of the disclosure into immune cells. Suitable vectors for use in accordance with the method of the present disclosure do not replicate in the subject's immune cells.

[00304] In one embodiment, the nucleic acid construct encoding the CAR according to the disclosure is introduced into an immune cell by a viral vector, particularly a lentiviral vector as described hereabove.

[00305] Alternatively, chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle).

[00306] Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise expose a cell to the inhibitor of the present disclosure, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the disclosure. Methods of testing a CAR for the ability to recognize target cells and for antigen specificity are known in the art. For instance, Clay et al, J. Immunol., 163: 507-513 (1999), teaches methods of measuring the release of cytokines (e.g., interferon-γ, granulocyte/monocyte colony stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α) or interleukin 2 (IL-2)). In addition, CAR function can be evaluated by measurement of cellular cytotoxicity, as described in Zhao et al, J. Immunol., 174: 4415-4423 (2005).

[00307] Once it is established that the transfected or transduced immune cell is capable of expressing the chimeric receptor as a surface membrane protein with the desired regulation and at a desired level, it can be determined whether the chimeric receptor is functional in the host cell to provide for the desired signal induction. Subsequently, the transduced immune cells can

be further reintroduced or administered to the subject to activate anti-tumor responses in the subject. To facilitate administration, the transduced T cells according to the disclosure can be made into a pharmaceutical composition or made into an implant appropriate for administration in vivo, with pharmaceutically acceptable carriers or diluents.

[00308] Once the cells expressing the CAR according to the disclosure are administered to a subject, the biological activity of the engineered cell populations and/or antibodies in some aspects is measured by any of a number of known methods. Parameters to assess include specific binding of an engineered or natural T cell or other immune cell to antigen, in vivo, e.g., by imaging, or ex vivo, e.g., by ELISA or flow cytometry. In certain embodiments, the ability of the engineered cells to destroy target cells can be measured using any suitable method known in the art, such as cytotoxicity assays described in, for example, Kochenderfer et al., J. Immunotherapy, 32(7): 689-702 (2009), and Herman et al. J. Immunological Methods, 285(1): 25-40 (2004). In certain embodiments, the biological activity of the cells also can be measured by assaying expression and/or secretion of certain cytokines, such as GM-CSF, IL-3, MIP-1α, TNF-α, IL- 10, IL-13, IFN-γ or IL-2.

[00309] In some aspects the biological activity is measured by assessing clinical outcome, such as reduction in tumor burden or load, stabilization of tumor, progression free survival, or overall survival.

[00310] Manufacturing methods contemplated herein may further comprise cryopreservation of modified immune cells for storage and/or preparation for use in a human subject. As used herein, "cryopreserving," refers to the preservation of cells by cooling to sub-zero temperatures, such as (typically) 77 K or −196° C. (the boiling point of liquid nitrogen).

[00311] In some embodiments, a method of storing genetically modified murine, human, or humanized CAR protein expressing immune effector cells which target an HLA-G expressing cell, comprises cryopreserving the immune effector cells such that the cells remain viable upon thawing. A fraction of the immune effector cells expressing the CAR proteins can be cryopreserved by methods known in the art to provide a permanent source of such cells for the future treatment of patients afflicted with an HLA-G expressing cancer cell. When needed, the cryopreserved transformed immune effector cells can be thawed, grown and expanded for more such cells.

[00312] Cryoprotective agents are often used at sub-zero temperatures to prevent the cells being preserved from damage due to freezing at low temperatures or warming to room

temperature. Cryopreservative agents and optimal cooling rates can protect against cell injury. Cryoprotective agents which can be used include but are not limited to dimethyl sulfoxide (DMSO) (Lovelock and Bishop, *Nature*, 1959; 183: 1394-1395; Ashwood-Smith, *Nature*, 1961; 190: 1204-1205), glycerol, polyvinylpyrrolidine (Rinfret, *Ann. N.Y. Acad. Sci.*, 1960; 85: 576), and polyethylene glycol (Sloviter and Ravdin, *Nature*, 1962; 196: 48).

Compositions and Formulations

[00313] The present disclosure also relates to a pharmaceutical or veterinary composition comprising the anti-HLA-G antibody or antibody fragment, the CAR, the nucleic acid construct, the vector and/or the cell as described hereabove.

[00314] In a particular aspect, the present disclosure relates to a pharmaceutical or veterinary composition comprising cells, preferably immune cells, comprising a CAR as described here above and/or comprising the nucleic acid construct encoding it as described hereabove. In one aspect, the pharmaceutical or veterinary composition may comprise a population of cells comprising a CAR specifically binding to β2M-associated HLA-G, preferably to both HLA-G1 and HLA-G5.

[00315] In a further aspect, the pharmaceutical or veterinary composition may comprise a first population of cells that express a CAR as described hereabove targeting β2M-associated HLA-G isoforms, and a second population of cells expressing a CAR that does not recognize HLA-G but recognized an antigen known to be a target of interest in CAR therapies such as anti-tumoral and/or anti-viral therapies. It will be understood that such second population of CAR expressing cells does not target HLA-G.

[00316] The present disclosure also relates to a pharmaceutical or veterinary composition containing a plurality of CAR-expressing cells of the disclosure, such as T cells and/or NK cells. The pharmaceutical composition may additionally comprise a pharmaceutically acceptable carrier, diluent or excipient. The pharmaceutical composition may optionally comprise one or more further pharmaceutically active polypeptides and/or compounds.

[00317] In one embodiment, the concentration of the cell expressing CAR according to the disclosure which is included in the pharmaceutical or veterinary composition is at least 0.001 mg/ml, at least 0.1 mg/ml, at least 0.5 mg/ml, at least 1 mg/ml, at least 5 mg/ml, at least 10 mg/ml, at least 15 mg/ml, at least 20 mg/ml, at least 25 mg/ml, at least 30 mg/ml, at least 35 mg/ml, at least 40 mg/ml, at least 45 mg/ml, at least 50 mg/ml, at least 55 mg/ml, at least 60 mg/ml, at least 65 mg/ml, at least 70 mg/ml, at least 75 mg/ml, at least 80 mg/ml, at least 85

mg/ml, at least 90 mg/ml, at least 95 mg/ml, at least 100 mg/ml, at least 105 mg/ml, at least 110 mg/ml, at least 115 mg/ml, at least 120 mg/ml, at least 125 mg/ml, at least 130 mg/ml, at least 135 mg/ml, at least 140 mg/ml, at least 150 mg/ml, at least 175 mg/ml, at least 200 mg/ml, at least 250 mg/ml, at least 275 mg/ml or at least 300 mg/ml.

[00318] In another embodiment, the concentration of the cell expressing CAR according to the disclosure which is included in the pharmaceutical or veterinary composition is between 0.001-0.01 mg/ml, between 0.01-0.1 mg/ml, between 0.1-1 mg/ml, between 1-10 mg/ml, between 10-50 mg/ml, between 50-100 mg/ml, between 50-150 mg/ml, between 50-200 mg/ml, between 50-250 mg/ml, between 50-300 mg/ml, between 100-200 mg/ml, between 100-300 mg/ml, or between 200-300 mg/ml.

[00319] In another embodiment, the pharmaceutical or veterinary composition comprises cells expressing the CAR according to the disclosure, particularly at least 100 cells, at least 200 cells, at least 400 cells, at least 500 cells, at least 700 cells, at least 1000 cells, at least 1500 cells, at least 2000 cells, at least 3000 cells, at least 5000 cells, at least 10,000 cells, at least 100,000 cells, at least 1 million cells, at least 10 million cells or at least 100 million cells expressing the CAR according to the disclosure.

[00320] In some embodiments, a pharmaceutical composition provided herein comprises a population of cells that express an anti-HLA-G CAR, wherein the population of cells comprises no less than 70% viable cells.

[00321] In some embodiments, a pharmaceutical composition provided herein comprises a population of cells that express an anti-HLA-G CAR, wherein the composition comprises no more than 5EU/kg endotoxin. The amount of endotoxin is measured by the European Pharmacopoeia 2.6.14 United States Pharmacopeia (USP) chapter <85> bacterial endotoxin test.

[00322] In some embodiments, a pharmaceutical composition provided herein comprises a population of cells that express an anti-HLA-G CAR, wherein the composition comprises less than 50 copies/μg replication-competent lentivirus. The amount of replication-competent lentivirus is measured by PCR.

[00323] In some embodiments, a pharmaceutical composition provided herein comprises a population of cells that express an anti-HLA-G CAR, wherein the cells comprise on average no more than 5 vector copies per transduced cell. The vector copy number is measured by ddPCR.

[00324] In some embodiments, a pharmaceutical composition provided herein comprises a population of cells that express an anti-HLA-G CAR, wherein (a) the population of cells comprises no less than 70% viable cells; (b) the composition comprises no more than 5EU/kg endotoxin; (c) the composition comprises less than 50 copies/μg replication-competent lentivirus; and (d) the cells comprise on average no more than 5 vector copies per transduced cell.

[00325] The pharmaceutical or veterinary composition according to the disclosure can be formulated for any conventional route of administration including a topical, enteral, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous or intraocular administration and the like.

[00326] Preferably, the pharmaceutical or veterinary composition according to the disclosure may be administered by enteral or parenteral route of administration. When administered parenterally, the pharmaceutical or veterinary composition according to the disclosure is preferably administered by intravenous route of administration. When administered enterally, the pharmaceutical or veterinary composition according to the disclosure is preferably administered by oral route of administration.

[00327] It will be understood by one skilled in the art that the formulations of the disclosure may be isotonic with human blood that is the formulations of the disclosure have essentially the same osmotic pressure as human blood. Such isotonic formulations generally have an osmotic pressure from about 250 mOsm to about 350 mOsm. Isotonicity can be measured by, for example, a vapor pressure or ice-freezing type osmometer. Tonicity of a formulation is adjusted by the use of tonicity modifiers. "Tonicity modifiers" are those pharmaceutically acceptable inert substances that can be added to the formulation to provide an isotonicity of the formulation. Tonicity modifiers suitable for this disclosure include, but are not limited to, saccharides, salts and amino acids.

[00328] Compositions and formulations for parenteral, intrathecal, or intraventricular administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carder compounds and other pharmaceutically acceptable carriers or excipients.

[00329] The pharmaceutical or veterinary composition according to the disclosure may further comprise a pharmaceutically acceptable vehicle. Thus, additional aspects of the disclosure relate to compositions comprising a carrier and one or more of the products-- e.g., a cell

comprising an anti-HLA-G CAR, a nucleic acid, a vector, an anti-HLA-G antibody or antibody fragment-- described in the embodiments disclosed herein. The formulations can be sterilized and, if desired, mixed with auxiliary agents such as carriers and excipients which do not deleteriously interact with the products– e.g., a cell comprising an anti-HLA-G CAR, a nucleic acid, a vector, an anti-HLA-G antibody or antibody fragment-- of the formulation.

[00330] Preferably, the pharmaceutical or veterinary compositions of the present disclosure including but not limited to any one of the claimed compositions may comprise CAR-expressing cells as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients as described hereafter. Desirably, a pharmaceutically acceptable form is employed which does not adversely affect the desired immune potentiating effects of recombinant cells according to the disclosure.

[00331] To facilitate administration, the transduced immune cells, preferably T cells and/or NK cells transduced with the nucleic acid construct encoding the CAR according to the disclosure can be made into a pharmaceutical composition for administration in vivo, with appropriate pharmaceutically acceptable carriers or diluents. The means of making such a composition have been described in the art (see, for instance, Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins, 21st edition (2005).

[00332] Particularly, formulations comprising populations of CAR-expressing cells may include pharmaceutically acceptable excipient(s). Excipients included in the formulations will have different purposes depending, for example, on the CAR construct, the subpopulation of cells used, and the mode of administration. The formulations comprising populations of CAR-expressing cells will typically have been prepared and cultured in the absence of any non-human components, such as animal serum (e.g., bovine serum albumin).

[00333] The formulation or composition may also contain more than one active ingredient useful for the particular indication, disease, or condition being treated with the binding molecules or cells, preferably those with activities complementary to the binding molecule or cell, where the respective activities do not adversely affect one another. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended.

[00334] The pharmaceutical or veterinary composition in some aspects can employ time-released, delayed release, and sustained release delivery systems such that the delivery of the composition occurs prior to, and with sufficient time to cause, sensitization of the site to be treated. Means known in the art can be used to prevent or minimize release and absorption of

the composition until it reaches the target tissue or organ, or to ensure timed-release of the composition. Such systems can avoid repeated administrations of the composition, thereby increasing convenience to the subject and the physician.

[00335] The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. As used herein "pharmaceutically acceptable carrier, diluent or excipient" includes without limitation any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, surfactant, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans or domestic animals. Exemplary pharmaceutically acceptable carriers include, but are not limited to, to sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; tragacanth; malt; gelatin; talc; cocoa butter, waxes, animal and vegetable fats, paraffins, silicones, bentonites, silicic acid, zinc oxide; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and any other compatible substances employed in pharmaceutical formulations.

[00336] In some embodiments, the compositions comprise an effective amount of anti-HLA-G CAR-expressing immune effector cells. As used herein, "an effective amount" of a genetically modified cell, *e.g.*, T cell, is the amount of cells required to achieve a beneficial or desired prophylactic or therapeutic result, including clinical results. The effective amount of a genetically modified therapeutic cell may vary according to factors such as the disease state, age, sex, and weight of the individual and includes an amount that is effective to "treat" a subject. The effective amount of the compositions described herein suitable for administration to a subject can be determined by a physician with consideration of individual differences in age, weight, extent of disease, and condition of the subject.

[00337] In some embodiments, a pharmaceutical composition comprising the T cells described herein may be administered at a dosage of 10^2 to 10^{10} cells/kg body weight, preferably 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. The number of cells will depend upon the ultimate use for which the composition is intended as will the type of cells included therein. The clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, or 10^{12} cells. CAR expressing cell compositions may be administered multiple times at dosages within these ranges. The cells may be allogeneic, syngeneic, xenogeneic, or autologous to the patient undergoing therapy. If desired, the treatment may also include administration of mitogens (*e.g.*, PHA), cytokines, and/or chemokines (*e.g.*, IFN-γ, IL-2, IL-12, TNF-alpha, IL-18, and TNF-beta, GM-CSF, IL-4, IL-13, Flt3-L, RANTES, MIP1α, *etc.*) as described herein to enhance induction of the immune response.

Subject, regimen and administration

[00338] The present disclosure relates to a pharmaceutical composition of the present disclosure or CAR expressing cells of the present disclosure for use as a medicament or for use for treating a disease or a disorder in a subject. It also relates to the use of a pharmaceutical composition of the present disclosure or CAR expressing cells of the present disclosure in the manufacture of a medicament for treating a disease or a disorder in a subject. Finally, it relates to a method for treating a disease or a disorder in a subject comprising administering a therapeutically effective amount of a pharmaceutical composition of the present disclosure or CAR expressing cells of the present disclosure to the subject.

[00339] The human subject according to the disclosure may be a human at the prenatal stage, a newborn, a child, an infant, an adolescent or an adult, in particular an adult at least 40 years old, an adult at least 50 years old, an adult at least 60 years old, or an adult at least 70 years old.

[00340] In some embodiments, the subject is a validated animal model for disease, adoptive cell therapy, and/or for assessing toxic outcomes such as cytokine release syndrome (CRS).

[00341] In some embodiments, the subject has persistent or relapsed disease, e.g., following treatment with another immunotherapy and/or other therapy, including chemotherapy, radiation, and/or hematopoietic stem cell transplantation (HSCT), e.g., allogeneic HSCT. In some embodiments, the administration effectively treats the subject despite the subject having become resistant to another therapy. In some embodiments, the subject has not relapsed but is

determined to be at risk for relapse, such as at a high risk of relapse, and thus the compound or composition is administered prophylactically, e.g., to reduce the likelihood of or prevent relapse.

[00342] In some embodiments, the disclosure includes the administration of CAR-expressing cells of the present disclosure or a composition containing CAR-expressing cells to a subject, such as one having, at risk for, or suspected of having a disease, condition or disorder. In some embodiments, the cells, and compositions are administered to a subject having a particular disease or condition to be treated, e.g., via adoptive cell therapy, such as adoptive T cell therapy. In some embodiments, the cells or compositions are administered to the subject, such as a subject having or at risk for a disease or condition. In some aspects, the methods thereby treat, e.g., ameliorate one or more symptom of the disease or condition. In some embodiments, the subject has been diagnosed with an immune disease, preferably a cancer. Diagnostic methods of autoimmune disease or cancer are well known by the man skilled in the art.

[00343] In some embodiments, the subject has already received at least one line of treatment, preferably several lines of treatment, prior to the administration of immune cells according to the disclosure or of a pharmaceutical or veterinary composition according to the disclosure.

[00344] Preferably, the treatment is administered regularly, preferably between every day and every month, more preferably between every day and every two weeks, more preferably between every day and every week, even more preferably the treatment is administered every day. In a particular embodiment, the treatment is administered several times a day, preferably 2 or 3 times a day, even more preferably 3 times a day.

[00345] The duration of treatment with the vector according to the disclosure, with the immune cells according to the disclosure or with a pharmaceutical or veterinary composition according to the disclosure is preferably comprised between 1 day and 20 weeks, more preferably between 1 day and 10 weeks, still more preferably between 1 day and 4 weeks, even more preferably between 1 day and 2 weeks. In some embodiments, the duration of the treatment is of about 1 week. Alternatively, the treatment may last as long as the disease persists.

[00346] The form of the pharmaceutical or veterinary compositions, the route of administration and the dose of administration of immune cells according to the disclosure or of a pharmaceutical composition according to the disclosure can be adjusted by the man skilled in the art according to the type and severity of the infection, and to the patient, in particular its age, weight, sex, and general physical condition. The compositions of the present disclosure

may be administered in a number of ways depending upon whether local or systemic treatment is desired.

[00347] In the case of adoptive cell therapy, methods for administration of cells for adoptive cell therapy are known and may be used in connection with the provided methods and compositions. For example, adoptive T cell therapy methods are described, e.g., in US Patent Application Publication No. 2003/0170238 to Gruenberg et al; U.S. Pat. No. 4,690,915 to Rosenberg; Rosenberg (2011) Nat Rev Clin Oncol. 8(10):577-85). See, e.g., Themeli et al. (2013) Nat Biotechnol. 31(10): 928-933; Tsukahara et al. (2013) Biochem Biophys Res Commun 438(1): 84-9; Davila et al. (2013) PLoS ONE 8(4): e61338.

[00348] One skilled in the art will recognize that, although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route. For example, intradermal delivery may be advantageously used over inhalation for the treatment of melanoma. Local or systemic delivery can be accomplished by administration comprising application or instillation of the formulation into body cavities, inhalation or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, intraportal, intrahepatic, peritoneal, subcutaneous, or intradermal administration.

[00349] Although systemic (intravenous, IV) injection is favored in clinical applications because of its ease of administration, several preclinical studies (Carpenito, et al. (2009) Proc. Natl. Acad. Sci. USA 106:3360-3365; Song, et al. (2011) Cancer Res. 71:4617-4627; Parente-Pereira, et al. (2011) J. Clin. Immunol. 31:710-718) suggest that the regional (intratumoral, IT or intraperitoneal, IP) administration of T cells may provide optimal therapeutic effects, which may be in part due to increased T cell trafficking to the tumor. For example, it has been shown that CAR T cells remain at the site of inoculation with minimal systemic absorption when delivered via IP or IT routes (Parente-Pereira, et al. (2011) J. Clin. Immunol. 31:710-718). In contrast, after intravenous administration, CAR T cells initially reach the lungs and then are redistributed to the spleen, liver, and lymph nodes. In addition, RNA CAR-electroporated T cells may be particularly suitable for regional administration, due to the transient nature of the CAR expression on the T cells (Zhao, et al. (2010) Cancer Res. 70:9053-9061). Furthermore, clinical studies have shown the feasibility and safety of both the intratumoral and intraperitoneal injection of T cells (Canevari, et al. (1995) J. Natl. Cancer Inst. 87:1463-1469; Duval, et al. (2006) Clin. Cancer Res. 12:1229-1236). Overall, a local route of administration of the recombinant T cells may provide the optimal therapeutic effect and decrease the potential

for the "on-target, off-organ" toxicity. Accordingly, in one embodiment, the CAR expressing cells according to the disclosure are administered locally, preferably by intra-tumoral and intraperitoneal injection.

[00350] The pharmaceutical composition in some embodiments contains the CAR cells in amounts effective to treat or prevent the disease or condition, such as a therapeutically effective or prophylactically effective amount. Therapeutic or prophylactic efficacy in some embodiments is monitored by periodic assessment of treated subjects. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful and can be determined. The desired dosage can be delivered by a single bolus administration of the composition, by multiple bolus administrations of the composition, or by continuous infusion administration of the composition. The amount of immune cells according to the disclosure or of a pharmaceutical composition according to the disclosure to be administered can be determined by standard procedure well known by those of ordinary skills in the art. Physiological data of the patient (e.g. age, size, weight and health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired) and the routes of administration have to be taken into account to determine the appropriate dosage, so as a therapeutically effective amount will be administered to the patient. Particularly, the appropriate dosages and dosing schedule can be based on clinical trials or well-established cell-based therapies (see, e.g., Topalian & Rosenberg (1987) Acta Haematol. 78 Suppl 1:75-6; US 4,690,915) or an alternate continuous infusion strategy can be employed.

[00351] In some embodiments, an effective amount or number of cells or pharmaceutical composition comprising those cells are administrated parenterally. In some embodiments, administration can be an intravenous administration. In some embodiments, administration can be directly done by injection within a tumor.

[00352] In certain embodiments, in the context of genetically engineered cells expressing the CARs, a subject is administered the range of about one million to about 100 billion cells, such as, e.g., 1 million to about 50 billion cells (e.g., about 5 million cells, about 25 million cells, about 500 million cells, about 1 billion cells, about 5 billion cells, about 20 billion cells, about 30 billion cells, about 40 billion cells, or a range defined by any two of the foregoing values), such as about 10 million to about 100 billion cells (e.g., about 20 million cells, about 30 million cells, about 40 million cells, about 60 million cells, about 70 million cells, about 80 million

cells, about 90 million cells, about 10 billion cells, about 25 billion cells, about 50 billion cells, about 75 billion cells, about 90 billion cells, or a range defined by any two of the foregoing values), and in some cases about 100 million cells to about 50 billion cells (e.g., about 120 million cells, about 250 million cells, about 350 million cells, about 450 million cells, about 650 million cells, about 800 million cells, about 900 million cells, about 3 billion cells, about 30 billion cells, about 45 billion cells) or any value in between these ranges, and/or such a number of cells per kilogram of body weight of the subject. For example, in some embodiments the administration of the cells or population of cells can comprise administration of about 103 to about 109 cells per kg body weight including all integer values of cell numbers within those ranges, for example, the cell compositions of the present disclosure can be administered in a dose, or dosages, where each dose comprises at least 10 cells/kg body weight, at least 100 cells/kg body weight; at least 1000 cells/kg body weight; at least 10,000 cells; at least 100,000 cells; at least 1 million cells; at least 10 million cells; at least 100 million cells; at least 1 billion cells or at least 10 billion cells/kg body weight.

[00353] Particularly, a sufficient number of the transduced immune cells will be introduced so as to achieve the desired therapeutic response. Desirably an effective amount or sufficient number of the isolated transduced cells is present in the composition and introduced into the subject such that long-term, specific, anti-tumor or anti-infectious agent responses are established to reduce the size or regrowth of a tumor or growth of an infectious agent than would otherwise result in the absence of such treatment. Desirably, the amount of transduced immune cells, preferably T cells, reintroduced into the subject causes a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 100% decrease in tumor size when compared to otherwise same conditions, wherein the transduced immune cells are not present.

[00354] A composition of the disclosure can be provided in unit dosage form wherein each dosage unit, e.g. an injection, contains a predetermined amount of the composition, alone or in appropriate combination with other active agents. The term unit dosage form, as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the composition of the disclosure, alone or in combination with other active agents, calculated in an amount sufficient to produce the desired effect, in association with a pharmaceutically acceptable diluent, carrier, or vehicle, where appropriate. The specifications for the novel unit dosage forms of the disclosure depend on the particular pharmacodynamics associated with the pharmaceutical composition in the particular subject.

[00355] The cells or population of cells can be administrated in one or more doses. In some embodiments, said effective amount or number of cells can be administrated as a single dose. In some embodiments, said effective amount or number of cells can be administrated as more than one dose over a period time. Timing of administration is within the judgment of managing physician and depends on the clinical condition of the patient. While individual needs vary, determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions within the skill of the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit.

[00356] For purposes of the disclosure, the amount or dose of the CAR material administered should be sufficient to generate a therapeutic or prophylactic response in the subject over a reasonable time frame. For example, the dose of the CAR material should be sufficient to bind to antigen, e.g. HLA-G isoform(s), or detect, treat or prevent disease in a period of from about 2 hours or longer, e.g., about 12 to about 24 or more hours, from the time of administration. In certain embodiments, the time period could be even longer. The dose will be determined by the efficacy of the particular CAR material and the condition of the subject, as well as the body weight of the subject to be treated.

[00357] For purposes of the disclosure, an assay, which comprises, for example, comparing the extent to which target cells are lysed or IFN-γ is secreted by T cells expressing the CAR, polypeptide, or protein upon administration of a given dose of such T cells to a mammal, among a set of mammals of which is each given a different dose of the T cells, could be used to determine a starting dose to be administered to a mammal. The extent to which target cells are lysed or IFN-γ is secreted upon administration of a certain dose can be assayed by methods known in the art.

Uses

Use in the treatment of disease or disorder and in combination therapy

[00358] In some aspects, the disease or disorder to be treated is a condition selected from a proliferative disease or disorder, preferably cancer; an infectious disease or disorder, preferably a viral infection; an inflammatory disease or disorder; and an immune disease or disorder, preferably autoimmunity or autoimmune diseases, allergies and graft-vs-host rejection. In some embodiments, the condition may be cancer.

[00359] The present disclosure relates to the use of an antibody, a cell, a nucleic acid construct, a vector and/or a pharmaceutical composition according to the disclosure for interfering or neutralizing the immune down-regulation due to HLA-G proteins in a host in need thereof.

[00360] In particular, the cell, the nucleic acid construct, the vector and/or the pharmaceutical composition according to the disclosure are particularly suitable for treatment of viral infections such as for example HIV-1, hepatitis B virus, and hepatitis C virus infections.

[00361] In particular, the cell, the nucleic acid construct, the vector and/or the pharmaceutical composition according to the disclosure are particularly suited for treatment of cancer, particularly of solid tumors or hematopoietic cancer, even more preferably when the availability of good selective single targets is limited.

[00362] The immune system can specifically identify and eliminate tumor cells based on their expression of tumor-specific antigens or molecules induced during malignant cell transformation. This process is referred to as tumor immune surveillance. Despite tumor immune surveillance, tumors can still develop in the presence of a functioning immune system. This occurs through tumor immunoediting, a process that comprises three major phases: 1) the elimination phase in which most immunogenic tumor cells are eliminated by cytotoxic T and NK cells; 2) the equilibrium phase in which tumor cells with reduced immunogenicity are selected; and 3) the escape phase in which variants that no longer respond to the host immune system are maintained (Urosevic and Dummer, 2008). HLA-G is involved in every phase of tumor immuno-editing by decreasing the elimination of tumor cells, by inhibiting the cytotoxic function of T and NK cells, and by trogocytosis, (i.e. the intercell transference of viable HLA-G molecules), which renders competent cytotoxic cells unresponsive to tumor antigens (LeMaoult et al., 2007; Caumartin et al., 2007). Therefore, the chimeric constructs of the present disclosure find application in subjects having or suspected of having a disease, disorder, or a particular condition, particularly subjects having or suspected of having a cancer. Particularly the chimeric constructs of the present disclosure find application in subjects having or suspected of having a cancer thereby reducing the size of a tumor or preventing the growth or re-growth of a tumor in these subjects or preventing the induction of an immunosuppressive microenvironment.

[00363] Accordingly, the present disclosure also relates to methods for inhibiting the growth of a tumor in a subject in need thereof and/or for treating a cancer patient in need thereof. The tumor may be a solid tumor, or a liquid tumor. In some embodiments, the tumor or cancer

expresses or overexpresses HLA-G. In certain embodiments, these methods comprise, or alternatively consist essentially of, or yet further consist of, administering to the subject or patient an effective amount of the isolated cell. In still further embodiments, the cell expressing a CAR according to the disclosure is a T cell or an NK cell. The isolated cell may be allogeneic or autologous to the subject or patient being treated. In a further aspect, the tumor expresses or overexpresses HLA-G antigen and the subject has been selected for the therapy by a diagnostic.

[00364] In one embodiment, the present disclosure relates to a method for reducing growth or preventing tumor formation in a subject by introducing a chimeric construct of the present disclosure into an immune cell, preferably a T cell or a NK cell, of the subject and reintroducing into the subject the transformed immune cell, thereby expressing the CAR according to the disclosure and effecting anti-tumor responses to reduce or eliminate tumors in the subject. The step of delivering the nucleic acid construct to the subject generally involves introducing a nucleic acid construct of the disclosure into an isolated immune cell (e.g., an autologous immune cell isolated from PBMC or immune cells derived from an allogeneic third party-derived immune cell donor) and introducing into the subject the transformed immune cell, thereby effecting antitumor responses to reduce or eliminate tumors in the subject, as in an adoptive T cell therapy method. For example, the immune cell may comprise a T cell and the subject is suffering from, or is believed to be suffering from, or is diagnosed as having tumor or cancer, e.g., a HLA-G expressing cancer. For example, the anti-HLA-G CAR molecules encoded by exemplary nucleic acid constructs of the present disclosure may be administered to the subject in the form of a recombinant immune cell engineered to express the anti-HLA-G CAR molecule.

[00365] CAR expressing cells according to disclosure and obtained by the methods described above, or cell lines derived from such cells, can be used as a medicament in the treatment of a disease, disorder, or condition in a subject. In some embodiments, such a medicament can be used for treating cancer.

[00366] In some embodiments, administering the treatment to the subject may comprise adoptive cell therapy (ACT) using immune cells harvested from the subject or from one or more donors. Accordingly, the cells can be cells that are xenogeneic, allogeneic or autologous to the subject. Generally, the cells are autologous to the subject.

[00367] In some embodiments, the cell therapy, e.g., adoptive cell therapy, e.g., adoptive T cell therapy, is carried out by autologous transfer, in which the cells are isolated and/or otherwise

prepared from the subject who is to receive the cell therapy, or from a sample derived from such a subject. Thus, in some aspects, the cells are derived from a subject, e.g., patient, in need of a treatment and the cells, following isolation and processing are administered to the same subject.

[00368] In some embodiments, the cell therapy, e.g., adoptive cell therapy, e.g., adoptive T cell therapy, is carried out by allogeneic transfer, in which the cells are isolated and/or otherwise prepared from a subject other than a subject who is to receive or who ultimately receives the cell therapy, e.g., a first subject. In such embodiments, the cells then are administered to a different subject, e.g., a second subject, of the same species. In some embodiments, the first and second subjects are genetically identical. In some embodiments, the first and second subjects are genetically similar. In some embodiments, the second subject expresses the same HLA class or supertype as the first subject. The cells of the present disclosure may be capable of killing target cells, such as cancer cells. The target cell may be recognizable by a defined pattern of antigen expression, for example the expression of antigen A or antigen B.

[00369] In some embodiments, ACT may comprise isolating primary immune cells from the subject or from one or more donors, transducing the primary immune cells with the nucleic acid construct or constructs of any of the foregoing embodiments, expressing the CAR in the transduced primary immune cells, and delivering the transduced immune cells into the subject. ACT may further comprise stimulating and/or expanding the immune cells prior to delivering the transduced immune cells to the subject.

[00370] For example, in some embodiments, ACT may comprise harvesting autologous or allogeneic T cells and transducing these T cells with one or more nucleic acid constructs, so that the T cells express a CAR mediating pro-inflammatory cytokine expression, and then infusing the cells into a subject in need thereof.

[00371] The disclosure also provides a method for treating cancer comprising delivering to a subject in need thereof an effective amount of the nucleic acid construct, a vector or vectors, or a transduced immune cell or pharmaceutical composition according to any of the foregoing embodiments, thereby treating the cancer. In some embodiments, the treatment of cancer may be measured by a decrease in tumor cell burden or by an increase in survival.

[00372] The disclosure additionally provides a method of immune therapy comprising administering to a subject a therapeutically effective amount of a nucleic acid construct or constructs, a vector or vectors, a recombinant cell or a pharmaceutical composition according

to any of the foregoing embodiments. Treatment with the cells of the disclosure may help prevent the escape or release of tumor cells which often occurs with standard approaches.

[00373] In certain embodiments, CAR expressing cells are modified in any number of ways, such that their therapeutic or prophylactic efficacy is increased. For example, the CAR may be conjugated either directly or indirectly through a linker to a targeting moiety. The practice of conjugating compounds, e.g., the CAR, to targeting moieties is known in the art. See, for instance, Wadhwa et al., J. Drug Targeting 1995;3(2): 111-127, and U.S. Pat. No. 5,087,616. Particularly, the present disclosure includes a type of cellular therapy where isolated cells are genetically modified to express CARs and the CAR cell is infused into a subject in need thereof. Such administration can promote activation of the cells (e.g., T cell activation) in a target-specific manner, such that the cells of the disease or disorder are targeted for destruction. In the case where the cell is a T cell, CAR T cells, unlike antibody therapies, are able to replicate in vivo resulting in long-term persistence that may lead to sustained control of targeted diseases, disorders, or conditions.

[00374] The CAR expressing cells as disclosed herein may be administered either alone or in combination with diluents, known anti-cancer therapeutics, and/or with other components such as cytokines or other cell populations that are immunostimulatory. They may be administered as a first line therapy, a second line therapy, a third line therapy, or further therapy.

[00375] In some embodiments, the cells expressing CAR are administered as part of a combination treatment, such as simultaneously with or sequentially with, in any order, another therapeutic intervention, such as an antibody or engineered cell or receptor or agent, such as a cytotoxic or therapeutic agent. The cells or antibodies in some embodiments are co-administered with one or more additional therapeutic agents or in connection with another therapeutic intervention, either simultaneously or sequentially in any order. In some contexts, the cells are co-administered with another therapy sufficiently close in time such that the cell populations enhance the effect of one or more additional therapeutic agents, or vice versa. In some embodiments, the cells or antibodies are administered prior to the one or more additional therapeutic agents. In some embodiments, the cells or antibodies are administered after to the one or more additional therapeutic agents, such as anti-cancer agents. An "anti-cancer" agent is capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or

a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer. More generally, these other compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cancer cells with the cell expressing the CAR according to the disclosure and the agent(s) or multiple factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the second agent(s).

[00376] In some embodiments, the cells expressing a CAR against HLA-G isoforms according to the disclosure are administered as part of a combination treatment such as simultaneously with or sequentially with, in any order, with other CAR-expressing cells that does not recognize HLA-G but are known to be useful in other CAR therapies such as anti-tumoral and/or anti-viral CAR therapies. Such CAR-expressing cells targets an antigen involved in a disease, preferably such as cancer or viral infection, preferably an antigen targeted in cancer therapies or in viral therapies. It will be understood that such antigen is not HLA-G.

[00377] In the context of the present disclosure, it is contemplated that cell therapy could be used similarly in conjunction with chemotherapeutic, radiotherapeutic, or immunotherapeutic intervention, as well as pro-apoptotic or cell cycle regulating agents such as immune checkpoint inhibitor.

[00378] Alternatively, the present therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and present disclosure are applied separately to the individual, one would generally ensure that a significant period of time did not expire between the times of each delivery, such that the agent and therapy would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several week (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

[00379] It is expected that the treatment cycles would be repeated if necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the cell therapy.

Targeted cancers

[00380] HLA-G is aberrantly expressed in many human solid malignant tumors in situ and malignant hematopoietic diseases including breast, ovarian, clear cell renal cell, colorectal, gastric, esophageal, lung, and hepatocellular cancers, as well as acute myeloid leukemia and chronic lymphocytic leukemia (B-CLL). The aberrant expression of HLA-G in malignant neoplasm is significantly correlated with poor clinical outcome of patients with colorectal cancer (CRC), gastric cancer (GC), non-small cell lung cancer (NSCLC), esophageal squamous cell cancer (ESCC), breast cancer, hepatocellular cancers, and B-CLL. Furthermore, serum soluble HLA-G is increased in various types of cancer patients (including patients with melanoma, acute leukemia, multiple myeloma, neuroblastoma, lymphoproliferative disorders, breast or ovarian cancer, non-small cell lung cancer, esophageal cancer, colorectal cancer; gastric cancer and hepatocellular carcinoma), when compared to normal healthy controls or benign disease cases.

[00381] Cancers that may be treated by the CAR-expressing cell, the nucleic acid construct, the vector or the pharmaceutical composition according to the disclosure include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors.

[00382] The CAR expressing cell, the nucleic acid construct, the vector or the pharmaceutical composition according to the disclosure may be used to treat cancers of the oral cavity and pharynx which includes cancer of the tongue, mouth and pharynx; cancers of the digestive system which includes esophageal, gastric and colorectal cancers; cancers of the liver and biliary tree which includes hepatocellular carcinomas and cholangiocarcinomas; cancers of the respiratory system which includes bronchogenic cancers, lung cancers and cancers of the larynx; cancers of bone and joints which includes osteosarcoma; cancers of the skin which includes melanoma; breast cancer; cancers of the genital tract which include uterine, endometrium, ovarian and cervical cancer in women, prostate and testicular cancer in men; cancers of the renal tract which include renal cell carcinoma and transitional cell carcinomas of the utterers or bladder; gastrointestinal stromal tumor, pancreas cancers, kidney cancers, colon cancers, cervix cancer, brain cancers including gliomas, glioblastoma multiform and medullobastomas; cancers of the endocrine system including thyroid cancer, adrenal carcinoma and cancers associated with multiple endocrine neoplasm syndromes; lymphomas including Hodgkin's lymphoma and non-Hodgkin lymphoma; B-cell lymphoma, monocytic lymphoma, marginal zone lymphoma, Burkitt's lymphoma, T and B lymphomas, Multiple Myeloma and plasmacytomas; leukemias both acute and chronic, prohemocytic leukemia, acute non-

lymphoblastic leukemia (ANLL), acute lymphoblastic leukemia (ALL), erythroleukemia, myeloid or lymphoid leukemia; and cancers of other and unspecified sites including neuroblastoma. In some embodiments, the cancer is selected from the group of Renal cell carcinoma (RCC), melanoma, kidney cancer and bladder cancer.

[00383] The cancers may comprise non solid tumors (such as hematological tumors, for example, leukemia and lymphoma) or may comprise solid tumors. As used herein, "solid tumor" is an abnormal mass of tissue that usually does not contain cysts or liquid areas. Solid tumors can be benign or malignant. Different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and lymphomas).

[00384] Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, neuroblastoma, retinoblastoma and brain metastases).

[00385] Hematologic cancers are cancers of the blood or bone marrow. Examples of hematological (or hematogenous) cancers include leukemias, including acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

[00386] In some embodiments, the cancer cells express or overexpress HLA-G. Preferably, the cancer cells express or overexpress HLA-G1 and/or HLA-G5. Preferably, when such cancer cells express these particular HLA-G isoforms, the CAR according to the disclosure specifically binds to HLA-G1 and HLA-G5, respectively.

Diagnostic and prognostic uses

[00387] The anti-HLA-G monoclonal antibodies or scFv disclosed herein are also useful in diagnostic and prognostic methods. As such, the present disclosure relates to the use of the antibodies disclosed herein in the diagnosis of HLA-G-related medical conditions in a subject.

[00388] The monoclonal antibodies or scFv disclosed herein are useful in methods known in the art relating to the localization and/or quantitation of a HLA-G polypeptide (e.g., for use in measuring levels of the HLA-G polypeptide within appropriate physiological samples, for use in diagnostic methods, for use in imaging the polypeptide, and the like). The monoclonal antibodies or scFv disclosed herein are useful in isolating a HLA-G polypeptide by standard techniques, such as western blotting, affinity chromatography methods for isolating cells or for flow cytometry-based cellular analysis or cell sorting or immunoprecipitation. A HLA-G antibody disclosed herein can facilitate the purification of natural HLA-G polypeptides from biological samples, e.g., mammalian sera or cells as well as recombinantly-produced HLA-G polypeptides expressed in a host system. Moreover, HLA-G monoclonal antibodies or scFv can be used to detect a HLA-G polypeptide (e.g., in plasma, a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the polypeptide. The HLA-G antibodies disclosed herein can be used diagnostically to monitor HLA-G levels in tissue as part of a clinical testing procedure, e.g., to determine the efficacy of a given treatment regimen. The detection can be facilitated by coupling (i.e., physically linking) the HLA-G antibodies disclosed herein to a detectable substance so as the HLA-G antibodies or fragments thereof are detectably labeled. The term "labeled", with regard to the antibody is intended to encompass direct labeling of the antibody by coupling (i.e., physically linking) a detectable substance to the antibody, as well as indirect labeling of the antibody by reactivity with another compound that is directly labeled. Non-limiting examples of indirect labeling include detection of a primary antibody using a fluorescently-labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently-labeled streptavidin.

[00389] The detection method of the present disclosure can be used to detect expression levels of HLA-G polypeptides in a biological sample in vitro as well as in vivo. In vitro techniques

for detection of HLA-G polypeptides include enzyme linked immunosorbent assays (ELISAs), Western blots, flow cytometry, immunoprecipitations, radioimmunoassay, and immunofluorescence (e.g., IHC). Furthermore, in vivo techniques for detection of HLA-G polypeptides include introducing into a subject a labeled anti-HLA-G antibody. By way of example only, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

[00390] In some aspects, HLA-G antibodies containing structural modifications that facilitate rapid binding and cell uptake and/or slow release are useful in in vivo imaging detection methods. In some aspects, the HLA-G antibody contains a deletion in the CH2 constant heavy chain region of the antibody to facilitate rapid binding and cell uptake and/or slow release. In some aspects, a Fab fragment is used to facilitate rapid binding and cell uptake and/or slow release. In some aspects, a F(ab)'2 fragment is used to facilitate rapid binding and cell uptake and/or slow release.

[00391] Accordingly, the present disclosure also provides prognostic (or predictive) assays for determining whether a subject is at risk of developing a medical disease or condition associated with increased HLA-G polypeptide expression or activity (e.g., detection of a precancerous cell). Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a medical disease or condition characterized by or associated with HLA-G polypeptide expression.

[00392] Another aspect of the present disclosure provides methods for determining HLA-G expression in a subject to thereby select appropriate therapeutic or prophylactic compounds for that subject.

[00393] Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing for developing cancer and/or solid tumors. Thus, the present disclosure provides a method for identifying a disease or condition associated with increased HLA-G isoform(s) expression levels in which a test sample is obtained from a subject and the HLA-G isoform(s) detected, wherein the presence of increased levels of HLA-G polypeptides compared to a control sample is predictive for a subject having or at risk of developing a disease or condition associated with increased HLA-G isoform(s) expression levels. In some aspects, the disease or condition associated with increased HLA-G isoform(s) expression levels is selected from the group consisting of for developing cancer and/or solid tumors.

[00394] In another embodiment, the present disclosure provides methods for determining whether a subject can be effectively treated with a compound for a disorder or condition associated with increased HLA-G expression wherein a biological sample is obtained from the subject and the HLA-G isoform(s) is/are detected using the HLA-G antibody or ScFv as described above. The expression level of the HLA-G polypeptide in the biological sample obtained from the subject is determined and compared with the HLA-G expression levels found in a biological sample obtained from a subject who is free of the disease. Elevated levels of the HLA-G in the sample obtained from the subject suspected of having the disease or condition compared with the sample obtained from the healthy subject is indicative of the HLA-G-associated disease or condition in the subject being tested.

[00395] There are a number of disease states in which the elevated expression level of HLA-G isoform(s) is known to be indicative of whether a subject with the disease is likely to respond to a particular type of therapy or treatment. Thus, the method of detecting HLA-G isoform(s) in a biological sample can be used as a method of prognosis, e.g., to evaluate the likelihood that the subject will respond to the therapy or treatment.

[00396] Further aspects of the present disclosure relate to methods for determining if a patient is likely to respond or is not likely to HLA-G CAR therapy. In specific embodiments, this method comprises contacting a tumor sample isolated from the patient with an effective amount of an HLA-G antibody and detecting the presence of any antibody bound to the tumor sample. In further embodiments, the presence of antibody bound to the tumor sample indicates that the patient is likely to respond to the HLA-G CAR therapy and the absence of antibody bound to the tumor sample indicates that the patient is not likely to respond to the HLA-G therapy. In some embodiments, the method comprises the additional step of administering an effective amount of the HLA-G CAR therapy to a patient that is determined likely to respond to the HLA-G CAR therapy.

[00397] Further aspects of the present disclosure relate to methods for determining if a patient is likely to respond or is not likely to HLA-G CAR therapy depending on the isoform(s) that is/are expressed by the tumor, particularly selected from HLA-G1, and HLA-G5. The identification of HLA-G expressed isoform(s) prior to treatment allowed the selection of the most suitable CAR that specifically binds HLA-G1 and HLA-G5 isoforms for use in an efficient treatment such as cell therapy.

Kits

[00398] Any of the compositions described herein may be included in a kit provided by the present disclosure. The kits will thus include, in suitable container means, recombinant/engineered cells of the present disclosure, and/or vectors encoding the nucleic acid constructs of the present disclosure, and/or nucleic acid constructs or related reagents of the present disclosure. In some embodiments, the kit further includes an additional agent for treating cancer or an infectious disease, and the additional agent may be combined with the nucleic acid construct(s) or cells, or other components of the kit of the present disclosure or may be provided separately in the kit. In some embodiments, means of taking a sample from an individual and/or of assaying the sample may be provided in the kit. In certain embodiments the kit includes cells, buffers, cell media, vectors, primers, restriction enzymes, salts, and so forth, for example. The kits may also comprise means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

[00399] The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. Such containers may include injection or blow- molded plastic containers into which the desired vials are retained. The kits of the present disclosure also will typically include a means for containing the components in close confinement for commercial sale. Such containers may include injection or blow molded plastic containers into which the desired vials are retained.

[00400] When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. The compositions may also be formulated into a syringe compatible composition. In which case, the container means may itself be a syringe, pipette, and/or other such like apparatus, from which the formulation may be applied to an infected area of the body, injected into an animal, and/or even applied to and/or mixed with the other components of the kit. However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

[00401] In some embodiments of the disclosure, cells that are to be used for cell therapy are provided in a kit, and in some cases the cells are essentially the sole component of the kit. The kit may comprise reagents and materials to make the desired cell. In specific embodiments, the reagents and materials include primers for amplifying desired sequences, nucleotides, suitable buffers or buffer reagents, salt, and so forth, and in some cases the reagents include vectors and/or DNA that encodes a CAR as described herein and/or regulatory elements therefor.

[00402] In some embodiments, there are one or more apparatuses in the kit suitable for extracting one or more samples from an individual. The apparatus may be a syringe, scalpel, and so forth.

[00403] In some cases of the disclosure, the kit, in addition to cell therapy embodiments, also includes a second cancer therapy, such as chemotherapy and/or immunotherapy, for example. The kit(s) may be tailored to a particular cancer for an individual and comprise respective second cancer therapies for the individual as described hereabove.

INCORPORATION BY REFERENCE

[00404] All references, articles, publications, patents, patent publications, and patent applications cited herein are incorporated by reference in their entireties for all purposes. However, mention of any reference, article, publication, patent, patent publication, and patent application cited herein is not, and should not be taken as, an acknowledgment or any form of suggestion that they constitute valid prior art or form part of the common general knowledge in any country in the world.

EXAMPLES

[00405] Although the foregoing embodiments have been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings contemplated herein that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Example 1 – Generation of humanized and chimeric LFTT-1 V_H and V_L variants

[00406] A series of humanized V_H and V_L amino acid sequences for LFTT-1 antibody were created by selectively backmutating certain humanized amino acid residues to murine amino

acid residues. Vernier zone residues, residues flanking the CDRs, and those important in variable region stability were backmutated in all designs. Humanness was defined either as the number of backmutations (with smaller numbers indicating a greater degree of humanness) or the degree of homology the IGVs share with human germline sequences. Exemplary humanized and chimeric LFTT-1 V_H and V_L variants are provided below in Table 5. The murine V_H and V_L parent sequences are designated as V_H0 and V_L0. V_H1 and V_L1 are most like the murine parent sequences whilst those designated as V_H5 and V_L5 are most similar to the optimal human template.

Table 5. Amino acid sequences of humanized LFTT-1 V_H and V_L variants

Domain or Region	Sequence	SEQ ID NO
V_H0 Heavy chain variable domain	QIQLVQSGPELKKPGETVKISCKASGYTFTTYGMSWVKQAPG KGLKWMGWIYTYSGVPTYADDLEGRFAFSLETSASTAYLQIN NLKNEDTATYFCARVRDGYYRYAMDYWGQGTSVTVSS	77
V_H1 Heavy chain variable domain	QIQLVQSGPELKKPGASVKISCKASGYTFTTYGMSWVKQAPG QGLKWMGWIYTYSGVPTYADDLTGRFVFSLETSVSTAYLQIS SLKAEDTATYFCARVRDGYYRYAMDYWGRGTSVTVSS	78
V_H2 Heavy chain variable domain	QIQLVQSGSELKKPGASVKISCKASGYTFTTYGMSWVKQAPG QGLKWMGWIYTYSGVPTYADDLTGRFVFSLETSVSTAYLQIS SLKAEDTATYFCARVRDGYYRYAMDYWGRGTLVTVSS	79
V_H3 Heavy chain variable domain	QIQLVQSGSELKKPGASVKVSCKASGYTFTTYGMSWVKQAPG QGLKWMGWIYTYSGVPTYADDLTGRFVFSLDTSVSTAYLQIS SLKAEDTATYFCARVRDGYYRYAMDYWGRGTLVTVSS	80
V_H4 Heavy chain variable domain	QIQLVQSGSELKKPGASVKVSCKASGYTFTTYGMSWVRQAPG QGLKWMGWIYTYSGVPTYADDLTGRFVFSLDTSVSTAYLQIS SLKAEDTATYYCARVRDGYYRYAMDYWGRGTLVTVSS	25
V_H5 Heavy chain variable domain	QIQLVQSGSELKKPGASVKVSCKASGYTFTTYGMSWVRQAPG QGLEWMGWIYTYSGVPTYADDLTGRFVFSLDTSVSTAYLQIS SLKAEDTATYYCARVRDGYYRYAMDYWGRGTLVTVSS	9
V_L0 Heavy chain variable domain	DIHMTQSPSSLSASLGGKVTITCKASQDINRYIAWYQHKPGK GPRLLIHFTSTLQSGIPSRFSGSGSGRDYSFSISNLEPEDIA TYYCLQYDDLRTFGGGTKLEIK	81
V_L1 Heavy chain variable domain	DIHMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQPGK APRLLIHFTSTLQSGIPSRFSGSGSGRDYTFTISSLQPEDIA TYYCLQYDDLRTFGQGTKLEIK	82
V_L2 Heavy chain variable domain	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQPGK APRLLIHFTSTLQSGIPSRFSGSGSGRDYTFTISSLQPEDIA TYYCLQYDDLRTFGQGTKLEIK	83
V_L3 Heavy chain variable domain	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQPGK APKLLIHFTSTLQSGIPSRFSGSGSGRDFTFTISSLQPEDIA TYYCLQYDDLRTFGQGTKLEIK	60
V_L4 Heavy chain variable domain	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQPGK APKLLIHFTSTLQSGVPSRFSGSGSGRDFTFTISSLQPEDIA TYYCLQYDDLRTFGQGTKLEIK	50
V_L5 Heavy chain variable domain	DIQMTQSPSSLSVSVGDRVTITCKASQDINRYIAWYQQQPGK APKLLIHFTSTLQSGVPSRFSGSGSGTDFTFTISSLQPEDIA TYYCLQYDDLRTFGQGTKLEIK	35

In the variable domains, CDR1, CDR2, CDR3, (from left to right) sequences are underlined; humanized residues are in bold; and backmutated residues are underlined/bold.

[00407] DNA constructs for the expression of humanized V_H or V_L for LFTT-1 variants were generated. Pairs of constructs (V_H and V_L) were used to co-transfect HEK 293 EBNA cells for the expression of humanized LFTT-1 variants. Construct pairs for humanized and chimeric full antibody expression are provided below in Table 6.

Table 6. Construct pairs of humanized LFTT-1 V_H and V_L for humanized and chimeric full antibody expression

LFTT-1 Variant	Antibody Composition	Heavy Chain Construct	Light Chain Construct
Chimeric LFTT-1	Chimeric HC and LC	LFTT-1 V_H0-hIgG1	LFTT-1 V_L0-hIg$_K$ C_L
V_H0/V_L1 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H0-hIgG1	LFTT-1 V_L1-hIg$_K$ C_L
V_H0/V_L2 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H0-hIgG1	LFTT-1 V_L2-hIg$_K$ C_L
V_H0/V_L3 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H0-hIgG1	LFTT-1 V_L3-hIg$_K$ C_L
V_H0/V_L4 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H0-hIgG1	LFTT-1 V_L4-hIg$_K$ C_L
V_H0/V_L5 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H0-hIgG1	LFTT-1 V_L5-hIg$_K$ C_L
V_H1/V_L0 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H1-hIgG1	LFTT-1 V_L0-hIg$_K$ C_L
V_H2/V_L0 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H2-hIgG1	LFTT-1 V_L0-hIg$_K$ C_L
V_H3/V_L0 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H3-hIgG1	LFTT-1 V_L0-hIg$_K$ C_L
V_H4/V_L0 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H4-hIgG1	LFTT-1 V_L0-hIg$_K$ C_L
V_H5/V_L0 LFTT-1	Chimeric HC/Humanized LC	LFTT-1 V_H5-hIgG1	LFTT-1 V_L0-hIg$_K$ C_L
V_H1/V_L1 LFTT-1	Humanized HC and LC	LFTT-1 V_H1-hIgG1	LFTT-1 V_L1-hIg$_K$ C_L
V_H1/V_L2 LFTT-1	Humanized HC and LC	LFTT-1 V_H1-hIgG1	LFTT-1 V_L2-hIg$_K$ C_L
V_H1/V_L3 LFTT-1	Humanized HC and LC	LFTT-1 V_H1-hIgG1	LFTT-1 V_L3-hIg$_K$ C_L
V_H1/V_L4 LFTT-1	Humanized HC and LC	LFTT-1 V_H1-hIgG1	LFTT-1 V_L4-hIg$_K$ C_L
V_H1/V_L5 LFTT-1	Humanized HC and LC	LFTT-1 V_H1-hIgG1	LFTT-1 V_L5-hIg$_K$ C_L
V_H2/V_L1 LFTT-1	Humanized HC and LC	LFTT-1 V_H2-hIgG1	LFTT-1 V_L1-hIg$_K$ C_L
V_H2/V_L2 LFTT-1	Humanized HC and LC	LFTT-1 V_H2-hIgG1	LFTT-1 V_L2-hIg$_K$ C_L
V_H2/V_L3 LFTT-1	Humanized HC and LC	LFTT-1 V_H2-hIgG1	LFTT-1 V_L3-hIg$_K$ C_L
V_H2/V_L4 LFTT-1	Humanized HC and LC	LFTT-1 V_H2-hIgG1	LFTT-1 V_L4-hIg$_K$ C_L
V_H2/V_L5 LFTT-1	Humanized HC and LC	LFTT-1 V_H2-hIgG1	LFTT-1 V_L5-hIg$_K$ C_L
V_H3/V_L1 LFTT-1	Humanized HC and LC	LFTT-1 V_H3-hIgG1	LFTT-1 V_L1-hIg$_K$ C_L
V_H3/V_L2 LFTT-1	Humanized HC and LC	LFTT-1 V_H3-hIgG1	LFTT-1 V_L2-hIg$_K$ C_L
V_H3/V_L3 LFTT-1	Humanized HC and LC	LFTT-1 V_H3-hIgG1	LFTT-1 V_L3-hIg$_K$ C_L
V_H3/V_L4 LFTT-1	Humanized HC and LC	LFTT-1 V_H3-hIgG1	LFTT-1 V_L4-hIg$_K$ C_L

V_H3/V_L5 LFTT-1	Humanized HC and LC	LFTT-1 V_H3-hIgG1	LFTT-1 V_L5-hIg$_K$ C_L
V_H4/V_L1 LFTT-1	Humanized HC and LC	LFTT-1 V_H4-hIgG1	LFTT-1 V_L1-hIg$_K$ C_L
V_H4/V_L2 LFTT-1	Humanized HC and LC	LFTT-1 V_H4-hIgG1	LFTT-1 V_L2-hIg$_K$ C_L
V_H4/V_L3 LFTT-1	Humanized HC and LC	LFTT-1 V_H4-hIgG1	LFTT-1 V_L3-hIg$_K$ C_L
V_H4/V_L4 LFTT-1	Humanized HC and LC	LFTT-1 V_H4-hIgG1	LFTT-1 V_L4-hIg$_K$ C_L
V_H4/V_L5 LFTT-1	Humanized HC and LC	LFTT-1 V_H4-hIgG1	LFTT-1 V_L5-hIg$_K$ C_L
V_H5/V_L1 LFTT-1	Humanized HC and LC	LFTT-1 V_H5-hIgG1	LFTT-1 V_L1-hIg$_K$ C_L
V_H5/V_L2 LFTT-1	Humanized HC and LC	LFTT-1 V_H5-hIgG1	LFTT-1 V_L2-hIg$_K$ C_L
V_H5/V_L3 LFTT-1	Humanized HC and LC	LFTT-1 V_H5-hIgG1	LFTT-1 V_L3-hIg$_K$ C_L
V_H5/V_L4 LFTT-1	Humanized HC and LC	LFTT-1 V_H5-hIgG1	LFTT-1 V_L4-hIg$_K$ C_L
V_H5/V_L5 LFTT-1	Humanized HC and LC	LFTT-1 V_H5-hIgG1	LFTT-1 V_L5-hIg$_K$ C_L

[00408] HLA-G binding efficiency of humanized LFTT-1 variants was assessed using a flow cytometry-based competition assay. Human HLA-G1 antigen expressing K562-HLAG1 cells and control K562 wild type cells were plated in the wells of 96-well U-bottomed plates (Corning, Amsterdam, The Netherlands) at 1E+6 cells/well. After washing the cells with DPBS + 10% FBS, titrations of each test antibody (83.33 nM to 0.04 nM in an 8 point three-fold dilution series) were conducted and the cells were resuspended before incubation in a static incubator at 37°C for one hour. Unbound antibody from the plates was removed by repeated centrifugation and washing steps with DPBS + 10% FBS before resuspending and incubating the cells with a fixed molarity (1.6 nM) of biotinylated murine LFTT-1 in a static incubator at 37°C for one hour. The cells were washed and resuspended with DPBS + 10 % FBS three additional times before resuspending with eBioscience™ Streptavidin PE Conjugate (Thermofisher, Loughborough, UK) and then incubated for an additional 30 min at 4°C. After a further wash, the plates were read on the Attune Flow Cytometer and recorded Mean Fluorescence intensity using the Attune YFP laser. To determine *IC50* values, the MFI for the mean values of technical replicates against test antibody molarities was plotted using Prism (Graphpad Software, La Jolla, USA). Background removal was conducted using MFI values determined using wild type K562 cells, followed by normalisation of the MFI readings so that the highest MFI for each series was defined as 100% in all cases and so that specific MFI was plotted against antibody molarity. *IC50* values were calculated using a fitted non-linear one-site *IC50* curve of the datapoints. *IC50* calculations for each LFTT-1 variant were performed for all plates to correct for plate-to-plate variation. The relative *IC50* of a given antibody variant is determined by division of the *IC50* value of the variant by that for the chimeric LFTT-1 run on the same plate. Variants with relative *IC50* values greater than 1 are predicted to have lower binding efficiency for K562-HLA-G1 cells, whilst those with values less than 1 are predicted

to have improved binding efficiency. Relative *IC50* values for LFTT-1 variants are provided in Table 7.

Table 7. Relative *IC50* values for LFTT-1 variants by heavy and light chain design

Relative IC_{50} values for LFTT-1 variants						
	V_H0 LFTT-1	V_H1 LFTT-1	V_H2 LFTT-1	V_H3 LFTT-1	V_H4 LFTT-1	V_H5 LFTT-1
V_L0 LFTT-1	N/A	0.72	2.95	3.20	-	-
V_L1 LFTT-1	1.46	1.35	2.05	0.96	0.77	1.48
V_L2 LFTT-1	0.98	1.66	1.27	0.96	1.44	1.47
V_L3 LFTT-1	0.93	1.66	0.67	0.99	2.71	3.18
V_L4 LFTT-1	1.3	0.61	1	0.78	1.14	0.95
V_L5 LFTT-1	1.65	0.92	1.29	0.89	0.79	1.1

[00409] Relative humanness of the LFTT-1 variants was determined by finding the homology of the heavy and light chain IGHV/IGKV and IGKJ-genes of each variant, to human germline sequences of the optimal human templates. This was achieved by concatenating the IGHV to IGHJ and IGKV to IGKJ amino acid sequences for all variants and likewise for the optimal human templates. Each variant's concatenated sequence was compared to the concatenated sequence for the templates in a pairwise alignment to determine percentage homology. Overall homology of the variants to the murine parent was calculated and backmutations were counted. FIG. 2 compares the humanness of LFTT-1 humanized variants, by homology (to parent and human germline) and by counting backmutations.

[00410] It is necessary to consider both the humanness and relative *IC50* scores for each antibody as there will often be a trade-off between binding efficiency and similarity to the human germline. Generally, more human-like designs lead to poorer binding efficiency as fewer murine residues are preserved in the designs, increasing the likelihood that the antibody will lose affinity for its target. More conservative designs generally lead to better binding efficiency. *IC50* scores for the humanized variants are plotted vs humanness in FIG. 3. Antibody variants with lower relative *IC50* scores and a greater degree of humanness make the best lead candidates for retention of antibody affinity for the target and tolerance by the human immune system. V_H4/V_L5 is a strong candidate in both respects.

[00411] MHC class II HLA-DR epitopes were detected by analyzing overlapping 9-mer peptides within the sequences of the humanized V_H and V_L regions of the LFTT-1 variants for their potential to bind to 51 HLA-DR alleles. The numbers of strong and medium 9-mer MHC class II binding peptides predicted to bind to HLA-DR in both chains in the whole V region versus the relative *IC50* values calculated for each variant are provided in Table 8.

Table 8. Number of HLA-DR epitopes in each humanized variant vs relative *IC50*

Antibody	Number of Strong and Medium HLA-DR Epitopes	Relative *IC50*
VH0/VL0 LFTT-1 (Chimeric)	23	-
VH1/VL1 LFTT-1 (Humanized)	21	1.35
VH1/VL2 LFTT-1 (Humanized)	20	1.66
VH1/VL3 LFTT-1 (Humanized)	20	1.66
VH1/VL4 LFTT-1 (Humanized)	20	0.61
VH1/VL5 LFTT-1 (Humanized)	20	0.92
VH2/VL1 LFTT-1 (Humanized)	21	2.05
VH2/VL2 LFTT-1 (Humanized)	20	1.27
VH2/VL3 LFTT-1 (Humanized)	20	0.67
VH2/VL4 LFTT-1 (Humanized)	20	1
VH2/VL5 LFTT-1 (Humanized)	20	1.29
VH3/VL1 LFTT-1 (Humanized)	18	0.96
VH3/VL2 LFTT-1 (Humanized)	17	0.96
VH3/VL3 LFTT-1 (Humanized)	17	0.99
VH3/VL4 LFTT-1 (Humanized)	17	0.78
VH3/VL5 LFTT-1 (Humanized)	17	0.89
VH4/VL1 LFTT-1 (Humanized)	17	0.77
VH4/VL2 LFTT-1 (Humanized)	16	1.44
VH4/VL3 LFTT-1 (Humanized)	16	2.71
VH4/VL4 LFTT-1 (Humanized)	16	1.14
VH4/VL5 LFTT-1 (Humanized)	16	0.79
VH5/VL1 LFTT-1 (Humanized)	17	1.48
VH5/VL2 LFTT-1 (Humanized)	16	1.47
VH5/VL3 LFTT-1 (Humanized)	16	3.18
VH5/VL4 LFTT-1 (Humanized)	16	0.95
VH5/VL5 LFTT-1 (Humanized)	16	1.1
Trastuzumab (Humanized)	11	-
Infliximab (Chimeric)	23	-

[00412] Table 8 shows that Infliximab (a chimeric antibody) contains approximately twice as many MHC class II binding 9-mers as Trastuzumab (a humanized antibody), consistent with their relative immunogenicity in the clinic. Furthermore, the murine LFTT-1 variable regions contain similar numbers of MHC class II binding 9-mers as Infliximab, whereas their humanized counterparts contain progressively fewer epitopes with the most 'human' variants containing 16 epitopes. Humanized LFTT-1 antibodies VH4/VL5 and VH5/VL4 exhibit low relative *IC50* values and a relatively high degree of humaneness. The number of strong and medium MHC class II binding 9-mers for VH4/VL5 and VH5/VL4 (16 each) is slightly higher than the humanized antibody Trastuzumab (11).

[00413] Based upon the relative *IC50* values, humanness and MHC class II assessment, three lead candidates for LFTT-1, VH4/VL5, VH5/VL4 and VH5/VL3 were selected. The properties of the three lead candidates are provided in Table 9.

Table 9. Comparison of 3 lead candidates for numbers of HLA-DRB epitopes, humanness, and binding efficiency

Humanized variant	Number of strong and medium HLA-DRB epitopes	Homology to human germline (%) - (Humanness)	Number of Backmutations (Humanness)	Relative *IC50*
LFTT-1 VH4/VL5	16	85.58	4	0.79
LFTT-1 VH5/VL4	16	85.58	3	0.95
LFTT-1 VH5/VL3	16	85.12	4	3.18

[00414] The kinetic constants of the three lead candidates for LFTT-1 were determined by the ligandTracer analysis according to the 2-to-1 bivalent binding model, with a weighted average of KD measurements (by Bmax) for both binding events determined for each candidate. The kinetic constants for each LFTT-1 lead candidate are provided in Table 10. The Ka (1/Ms, on rate) is the association constant and is a measure of the rate of complex formation between the HLA-G1 expressing cells and the antibodies under investigation. The Kd (1/s, off rate) is the dissociation constant and is a measure of the rate at which the complex dissociates. The KD (M) is the dissociation constant for the antibody-antigen complex, determined by division of the Kd by the Ka. LigandTracer revealed that all three antibodies bound cells expressing human HLA-G1 with KD values in the low nanomolar range.

Table 10. Kinetic constants for the LFTT-1 lead candidates determined by LigandTracer

Lead Antibody Candidate	Bmax1	Bmax2	Ka1 (1/Ms)	Ka2 (1/Ms)	Kd1 (1/s)	Kd2 (1/s)	KD1 (M)	KD2 (M)	KD (Weighted Average (M)	Chi^2
LFTT-1 V_H0/V_L0 (Chimeric)	55.65	10.02	1.85E+05	7.01E+05	8.68E-04	1.89E-05	4.70E-09	2.70E-11	3.99E-09	0.23
LFTT-1 V_H4/V_L5	99.45	42.26	1.49E+05	2.69E+05	5.85E-04	3.14E-06	3.93E-09	1.17E-11	2.76E-09	9.73
LFTT-1 V_H5/V_L4	76.39	32.57	1.37E+05	2.97E+05	6.45E-04	3.10E-06	4.71E-09	1.04E-11	3.31E-09	3.06
LFTT-1 V_H5/V_L3	100.27	31.92	1.19E+05	2.96E+05	6.92E-04	2.70E-06	5.80E-09	9.11E-12	4.40E-09	3.81

Example 2 – Assessment of the binding profile of humanized LFTT-1 V_H4/V_L5 using a human plasma membrane protein cell array

[00415] The Retrogenix Cell Microarray Technology was used to screen for specific off-target binding interactions of a humanized mouse IgG1 antibody known as hLFTT-1 V_H4/V_L5.

[00416] 2, 5 or 20 µg/mL of hLFTT-1 VH4/VL5, 1 µg/mL Rituximab biosimilar, or PBS alone was added to slides of untransfected HEK293 cells (areas outside the spots) and HEK293 cells over-expressing HLA-G, HLA-G + B2M, CD20 or EGFR (spotted areas) after or before fixation. Slides were subsequently incubated with an AF647 anti-hIgG Fc detection antibody

followed by fluorescence imaging. hLFTT-1 V_H4/V_L5 showed a specific interaction with HLA-G + β2M, the primary target, on both fixed and live cell microarrays. Significant interactions with HLA-G and EPHB6 were observed on the live cell microarray only.

[00417] The interactions for hLFTT-1 V_H4/V_L5 with HLA-G, HLA-G + β2M and EPHB6 were confirmed in a single-dose flow cytometry follow on study. Human HEK293 cells were transfected with expression vectors encoding ZsGreen1 only, or ZsGreen1 and HLA-G, HLA-G + β2M, EPHB6 or CD20 (assay control). Live cell transfectants were incubated with 5 µg/mL hLFTT-1 VH4/VL5, 1 µg/mL Rituximab biosimilar (assay control) or assay buffer only. Cells were washed and incubated with the same AF647 anti-hIgG Fc detection antibody as used in the cell microarray screens. Cells were again washed and analysed by flow cytometry using an Accuri flow cytometer (BD). A 7AAD live/dead dye was used to exclude dead cells in the analysis, and ZsGreen+ (transfected) cells were selected for analysis. hLFTT-1 VH4/VL5 showed medium (8.2 mean fold) and strong (69.5 mean fold) binding intensity with primary targets HLA-G and HLA-G + β2M, respectively. Strong (13.9 mean fold) binding intensity was also observed with EPHB6. The binding results for hLFTT-1 V_H4/V_L5 with target antigens are provided in Table 11.

Table 11. The flow cytometry results for the interaction of hLFTT-1 V_H4/V_L5 with HLA-G, HLA-G + B2M and EPHB6

	Median AF647 Fluorescence (Fold change from ZsGreen-only transfected HEK293s)															
	UCN6271 HLA-G NM_002127.5				*UCN6312 HLA-G + B2M NM_002127.5 + DQ890551*				*UCN1827 EPHB6 NM_004445.3*				*CD20*		*HEKs (ZsGreen-only)*	
	n=1		*n=2*		*n=1*		*n=2*		*n=1*		*n=2*		*n=1*		*n=1*	*n=2*
	M	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	
hLFTT-1 VH4/VL5	5206	7.8	5720	8.6	44544	67.0	47903	72.0	9880	14.9	8552	12.9	747	1.1	644	687
Assay buffer	617	0.9	664	1.0	582	0.9	607	0.9	649	1.0	664	1.0	792	1.2	635	680
Rituximab biosimilar	663	0.9	NT	NT	624	0.9	NT	NT	629	0.9	NT	NT	381463	538	709	NT

Fold Change from ZsGreen-1 transfected HEKs	
2.0 – 2.5	Low Confidence Very weak binding intensity
2.6 – 4.0	Weak binding intensity
4.1 – 10	Medium binding intensity
> 10	Strong binding intensity

NT	Not tested
M	Median
F	Fold

[00418] In a further dose-response follow-on study, *EC50* values could not be determined on transiently transfected cells, as the binding with HLA-G and HLA-G + β2M showed a biphasic profile, and the binding to EPHB6 was not maximal at 300 µg/mL, the highest dose tested. It is unlikely that the interaction with EPHB6 is physiologically relevant with an *EC50* of >300 µg/mL. The binding results are provided in Table 12.

Table 12. The flow cytometry results for the interaction of hLFTT-1 V_H4/V_L5 with HLA-G, HLA-G + β2M and EPHB6 in a dose-response study

Transfectant		*Median AF647 Fluorescence (Fold Change from corresponding ZsGreen1-only transfectants)*									
		hLFTT-1 VH4/VL5 (µg/mL)									*Rituximab (µg/mL)*
		0	*0.018*	*0.07*	*0.3*	*1.2*	*4.7*	*18.75*	*75*	*300*	*1*
UCN 6271 HLA-G NM_002127.5	Median	568	4014	4124	5617	5730	5848	6305	8116	13320	564
		NT	3995	3944	5418	5652	5747	6758	8061	12356	NT
	Fold Change	0.9	6.5	6.7	8.9	9.3	8.7	7.1	5.0	2.6	0.9	
		NT	6.5	6.4	8.6	9.2	8.5	7.6	5.0	2.5	NT
UCN 6312 HLA-G + B2M NM_002127.5+ DQ890551	Median	606	19806	34245	41071	42760	47144	50749	62555	79354	568
		NT	20188	34044	41220	43576	47180	52512	60107	79816	NT
	Fold Change	1.0	32.1	55.2	65.3	69.2	69.8	57.1	38.6	15.8	0.9
		NT	32.7	54.9	65.6	70.6	69.9	59.1	37.1	15.9	NT
UCN 1827 EPHB6 NM_004445.3	Median	663	1074	1612	2647	5561	16840	44204	144541	475378	623
		NT	1126	1727	2832	6602	17628	53254	162196	443903	NT
	Fold Change	1.1	1.7	2.6	4.2	9.0	24.9	49.8	89.3	94.5	1.0
		NT	1.8	2.8	4.5	10.7	26.1	59.9	100.2	88.3	NT
CD20	Median	732	NT	NT	NT	NT	NT	NT	NT	NT	268578
	Fold Change	1.2	NT	NT	NT	NT	NT	NT	NT	NT	431.1
ZsGreen-only transfectants	Median	598	624	610	618	612	666	894.	1724	5030	623
		NT	611	630	639	623	684	883	1515	3549	NT

[00419] In a further dose-response experiment, HLA-G and HLA-G + β2M transiently transfected cells, as well as stably transfected HEK-HLA-G1 cells were incubated with a dose-range of hLFTT-1 V_H4/V_L5 or commercial anti-HLA-G antibody to determine *EC50* values for the primary targets HLAG and HLA-G + β2M, and to determine whether a commercial anti-human HLA-G antibody also shows a biphasic binding profile with the primary targets. hLFTT-1 V_H4/V_L5 showed a biphasic binding profile with the primary targets, HLA-G, HLA-G + β2M and stably transfected HEK-HLA-G1 cells. At the 2 top doses (167 and 500 µg/mL)

high background binding was observed with ZsGreen only transfected cells, which suggests that at these very high concentrations, the test antibody is interacting with an endogenously produced protein on HEK293 cells. In target transfected cells, the first inflection point is therefore the interaction with the transfectant, and the second inflection point is the interaction with the endogenous HEK cell protein (on either HEK293 cells or HEK-HLA-G1 cells). After excluding the 167 and 500 µg/mL doses, *EC50* values of 0.35, 0.18 and 0.05 µg/mL were calculated for HLA-G, HLA-G + β2M and HEK-HLA-G1 stably transfected cells respectively. The binding results for hLFTT-1 V_H4/V_L5 with target antigens are provided in Table 13.

Table 13. The flow cytometry results for the interaction of the test antibody with HLA-G, HLA-G + β2M and EPHB6 in a dose-response study

Transfectant		*Median AF647 Fluorescence (Fold Change from corresponding ZsGreen1-only transfectants)*											
		hLFTT-1 VH4/VL5 (µg/mL) +AF647 anti-human IgG Fc											
		0	*0.0085*	*0.025*	*0.076*	*0.23*	*0.69*	*2.1*	*6.2*	*18.5*	*55.6*	*167*	*500*
UCN 6271 HLA-G NM_002127.5	**Median**	1268	4606	7139	8769	12739	15989	15828	19048	19725	26809	39764 *	61055 *
		1208	3959	5941	8833	11614	12420	13407	16011	18243	26733	41681 *	64657 *
	Fold Change	1.3	4.5	7.2	8.8	12.2	14.8	13.0	12.5	8.1	5.9	3.8	2.7
		1.3	3.8	6.0	8.9	11.3	11.5	11.0	10.5	7.5	5.9	4.0	2.9
UCN 6312 HLA-G + B2M NM_002127.5 + DQ890551	**Median**	1048	5689	9961	13767	33254	42876	44680	47162	53824	61670	78478 *	96676 *
		961	5872	10692	20703	31513	41074	44464	47973	50925	60565	79623 *	106313*
	Fold Change	1.1	5.5	10.1	13.9	31.8	39.8	36.8	31.0	22.2	13.5	7.6	4.3
		1.0	5.7	10.8	20.9	30.2	38.1	36.6	31.5	21.0	13.3	7.7	4.7
		924	1046	1016	1016	1102	1140	1246	1531	2541	4869	10878	24111
		996	1019	966	969	988	1015	1,185	1513	2300	4235	9821	21040
HEK-HLA-G1 Cells	**Median**	962	22385	39920	79113	108322	117572	120755	129822	137403	149556	167153*	200138*
		1036	21055	44648	87887	128463	115075	120441	126528	133924	145048	161868*	184531*

Fold Change from ZsGreen-1 transfected HEKs	
2.0 – 2.5	Low Confidence Very weak binding intensity
2.6 – 4.0	Weak binding intensity
4.1 – 10	Medium binding intensity
> 10	Strong binding intensity
NT	Not tested

* Data point not included in EC_{50} analysis

[00420] The commercial anti-human HLA-G Antibody (Clone 87G) did not show a biphasic profile and *EC50* values of 1.6, 3.1 and 1.9 µg/mL were calculated for the interactions with HLA-G, HLA-G + β2M and the stable HEK-HLA-G1 cells respectively. The *EC50* values of hLFTT-1 V_H4/V_L5 and Clone 87G are provided in Table 14.

Table 14. The *EC50* values for the interaction of the test antibody hLFTT-1 V_H4/V_L5 and Clone 87G with HLA-G, HLA-G + β2M and HLA-G1

	EC_{50} (μg/mL) (95% CI)	
	hLFTT-1 V_H4/V_L5	***anti-human HLA-G Antibody (Clone 87G)***
HLA-G	***0.35*** *(0.07 to 2.2)*	***1.6*** *(1.1 to 2.3)*
HLA-G + B2M	***0.18*** *(0.12 to 0.29)*	***3.1*** *(2.1 to 4.6)*
HEK-HLA-G1 Cells	***0.05*** *(0.03 to 0.08)*	***1.9*** *(1.2 to 2.9)*

Example 3 – Preclinical assessment of CAR-T efficacy in solid tumors

Cytolytic function of humanized anti-HLA-G LFTT-1 CAR-T in vitro

[00421] To evaluate the specific cytotoxicity against solid tumors *in vitro*, humanized LFTT-1 CAR-T cells were incubated with multiple chemo resistant SKOV-3-HLA-G+ cell line at the ratio of 12:1, 6:1 and 3:1. FIG. 4 shows the percentage of tumor cell lysis after incubation of the CAR-T cells. It was observed that anti-HLA-G CAR-T cells displays specific cytotoxicity against multiple chemo resistant SKOV-3-HLA-G+ cell lines in a dose dependent manner while shows minimal cytotoxicity against HLA-G negative cells.

Ex vivo infiltration and activation of humanized anti-HLA-G LFTT-1 CAR-T

[00422] To evaluate the degree of infiltration of humanized anti-HLA-G LFTT-1 CAR-T cells into solid tumor tissues, immunohistochemistry (IHC) was performed on renal cell carcinoma tissues after incubation with anti-HLA-G CAR-T cells. Strong and specific *ex vivo* activation of anti-HLA-G CAR-T cells was observed upon infiltration of clear cell Renal Cell Carcinoma (ccRCC) human biopsies and RCC-HLA-G+ tumor models, respectively. FIG. 5A is a representative IHC image of anti-HLA-G CAR-T cells on a human ccRCC tissue sample. A considerable number of anti-HLA-G CAR-T cells were observed on the human ccRCC tissue sample. FIG. 5B displays the activation ratio of the CAR-T cells compared to the non-transduced control cells. There is a significant increase in the action of CAR-T cells compared to non-transduced control cells. FIG. 5C summarizes the activation ratios of the CAR-T cells on PDX RCC and human ccRCC, respectively. It was observed that anti-HLA-G CAR-T cells were significantly activated in PDX RCC and human ccRCC tissue samples that express HLA-

G. The results of the *ex vivo* assay for CAR-T cells may provide predictive values for *in vivo* efficacy.

In vivo functions of humanized anti-HLA-G LFTT-1 CAR-T cells

[00423] To investigate *in vivo* functions of humanized anti-HLA-G LFTT-1 CAR-T cells, NGS mice were implanted with renal cell carcinoma patient derived xenografts (RCC PDX) followed by inoculation of humanized anti-HLA-G LFTT-1 CAR-T cells on day 22 (FIG. 6A). The cytotoxicity of anti HLA-G CAR-T cells against HLA-G tumor cells were monitored. Significant therapeutic efficacies of anti-HLA-G LFTT-1 CAR-T cells were observed. FIG. 6B shows the measurements of the tumor volume in NGS mice after tumor implantation over 79 days. Substantially more effective control and elimination of PDX primary tumor was observed in NGS mice with inoculation of humanized anti-HLA-G LFTT-1 CAR-T cells than the control T cells. These results demonstrate significant therapeutic efficacies of anti-HLA-G LFTT-1 CAR-T cells in an *in vivo* renal cancer system.

Example 4 –GMP manufacture of anti-HLA-G CAR-T cells

[00424] The present disclosure provides a closed, continuous, and automated process for the GMP (good manufacturing process) manufacturing of the anti-HLA-G CAR-T cell final drug product (FDP). This process has been adapted from the Miltenyi Prodigy TCT Process. The formulated cryopreserved product is designated as the FDP.

[00425] All raw materials are GMP grade from qualified vendors. The manufacturing of anti-HLA-G CAR-T cells is supported by a qualified contract development and manufacturing organization (CDMO) and operates under a quality agreement. All materials are examined/tested according to the standard operating procedures prior to its use. Critical raw materials are presented in Table 15.

Table 15. Critical raw materials used for anti-HLA-G CAR-T cell cGMP manufacturing process

Starting material, raw material or reagent	Source A= animal R= recombinant S= synthetic H= human	Compliance
Lentivirus	R	cGMP*
PBMC/Leukopak	H	cGMP
MACS GMP Media	S	cGMP
MACS GMP Cytokines (IL7 and IL15)	R	cGMP
CliniMACS CD4 GMP MicroBeads	S	cGMP

CliniMACS CD8 GMP MicroBeads	S	cGMP
MACS GMP T Cell Trans Act	R/S	cGMP
Human Serum Albumin (HSA)	H	cGMP
Cryostor®	S	cGMP

* cGMP: current Good Manufacturing Process

[00426] Anti-HLA-G CAR-T cell product is manufactured from autologous peripheral blood leukapheresis source material. Leukapheresis is performed at clinical site(s), with specific leukapheresis equipment, by experienced and authorised staff according to local standard clinical procedures. The donors are tested for applicable infectious diseases and in addition, donors must be assessed for suitability prior to the apheresis procedure, according to clinical protocol. A certificate of analysis is provided to certificate Leukapheresis testing and quality. These procedures are also in compliance with European Directive 2006/17/EC and Directive 2004/23/EC.

[00427] Using CliniMACS® Prodigy-TCT manufacturing processes, the anti-HLA-G CAR expression and CAR-T cell expansion were evaluated on peripheral blood mononuclear cells (PBMCs) isolated from 4 different healthy donors. Anti-HLA-G CAR-T cells were identified as CD3+CD19+ double positive population by flow cytometry. Anti-HLA-G CAR-T cells can be produced (LVV MOI=2) in large scale (4-5x10^9cells) with a transduction efficacy ranging from 69% to 85% of T cells expressing the CAR construct (CD3+CD19+ double positive) and a viability higher than 90%. Table shows the characterization of anti-HLA-G CAR CD4/CD8 T cell composition from $CD3^+$ cells depending on lentiviral MOI used.

Table 16. Determination of anti-HLA-G CAR CD4/CD8 T cell composition from $CD3^+$ cells depending on lentiviral MOI used

TCT run	MOI	$CD3^+$ TCT process	Transduction at day 5	Transduction at day 12	Total $CD45^+$ at day 12	CAR-T^+ $CD4^+$	CAR-T^+ $CD8^+$
N=4	2	60 to 100x10^6	68 to 85%	69 to 85%	4.4 to 5.4x10^9	58 to 80%	12 to 41%

[00428] Given these primary non-clinical results, anti-HLA-G CAR-T cells can be produced in large quantities with a transduction efficacy ranging from 69 to 85% of T cells expressing the CAR construct at a low MOI.

[00429] Anti-HLA-G CAR-T cells consist of autologous CD3+ T cell, with typically make up >90% of the total cell population. Product-related impurities are cellular impurities derived from leukapheresis material such as red blood cells, granulocytes, dead cells, and B cells/B-lineage lymphoblasts. Typical percentage of cellular impurities make <2% of the overall anti-

HLA-G CAR-T cell final product. Cell viability is controlled during the manufacturing and formulation process.

[00430] Ancillary materials and reagents that are not intended to be present in the final product are evaluated as part of the final drug product characterization testing.

[00431] The quality of anti-HLA-G CAR-T cell product is assessed using the product release quality control testing plan (Table 17). Analytical test methods for the assessment of anti-HLA-G CAR-T cell product critical quality attributes in support of process development/optimization, raw materials selection, intermediate, drug substance (day 12 harvest) and final product characterization (FDP formulated and cryopreserved) are summarized in Table 17.

Table 17. anti-HLA-G CAR-T cell product release QC testing plan

Category	Test	Method	Sampling Point	Specification
Enriched T cell				
Release	Total Viable cells	Automated cell counter	Cryopreserved sample	Report result
Release	Viability	Automated cell counter	Cryopreserved sample	≥70%
Release	%$CD3^+$ Cells	Flow cytometry	Cryopreserved sample	Report result
Product characterization**	Immunophenotyping, Multilineage panel	Flow cytometry	Cryopreserved sample	Report result
Product characterization**	Immunophenotyping, T cell panel	Flow cytometry	Cryopreserved sample	Report result
CAR-T final product				
In Process	%CAR^+T cells	Flow cytometry	Fresh sample: Day to be finalized	TBD
Release	Sterility	BD BACTEC*	Formulated sample	No growth
Release	Mycoplasma	PCR*	Harvest sample	Negative
Release	Endotoxin	Eur. Ph. 2.6.14 USP <85>	Formulated sample	≤5EU/kg
Release	RCL	PCR	Cryopreserved sample	< 50 copies/ µg DNA
Release	Total viable cells	Automated cell counter	Cryopreserved sample	Report result
Release	Viability	Automated cell counter	Cryopreserved sample	≥70%
Release	%CAR^+T cells	Flow cytometry	Cryopreserved sample	TBD
Release	Number of %CAR^+T cells	Calculated value	Cryopreserved sample	Report result

Category	Test	Method	Sampling Point	Specification
Release	Vector copy number	ddPCR	Cryopreserved sample	≤5 copies/ transduced cells
Product Characterization**	Immunophenotyping T cell panel	Flow Cytometry	Cryopreserved sample	NA
Product Characterization**	Immunophenotyping Multilineage panel	Flow Cytometry	Cryopreserved sample	NA
Product Characterization**	Cytokine secretion (IFNgamma)	Cell-stimulated ELISA	Cryopreserved sample	NA

* Alternative method compliant to Eur. Ph. 2.6.7, USP <63>, Eur. Ph. 2.6.1 and USP <71>
** Characterization Testing Only. Not require for release testing

[00432] The objective of the product release QC testing plan is to develop a strategy that allows the reduction in time for the release testing (to about 2 weeks) of the anti-HLA-G CAR-T cell FDP while maintaining all the safety and testing requirements for the FDP release prior to patient dosing.

[00433] Stability studies are conducted on the GMP lot of anti-HLA-G CAR-T cell FDP. The purpose of these studies is to obtain long-term stability information. Stability assessment and testing plans are described in Table below.

Table 18. Anti-HLA-G CAR-T cell drug product stability Program Plan

Stability	Assay	0 month (Release)	1 month	3 months	6 months
Enriched T cells*	Sterility	X	-	-	X
	Container Integrity	X	-	X	X
	Total Viable cells	X	-	X	X
	Cell viability	X	-	X	X
CAR-T cells*	Sterility	X	-	-	X
	Container Integrity	X	X	X	X
	Total Viable cells	X	X	X	X
	Cell viability	X	X	X	X

*One representative batch; an in-use stability and an administration device study will also be conducted on CAR-T cells.

[00434] The potency of GMP grade anti-HLA-G CAR-T cells derived from three donors was assessed by an *in vitro* cytotoxicity assay. Anti-HLA-G CAR-T cells were incubated on LCL cells expressing GFP and HLA-G (LCL-GFP-HLA-G). FIGS. 7A-7C show that GMP grade anti-HLA-G CAR-T cells derived from the three different donors lysed HLA-G-expressing cells in a dose-dependent manner, and caused significantly greater cell death than non-transduced (NTD) CAR-T cells did. The levels of IFN-γ secreted by anti-HLA-G CAR-T cells derived from the three donors were measured using the Mesoscale Discovery (MSD) multiplex analysis of cytokines. FIGS. 8A-8C show that anti-HLA-G CAR-T cells secreted significantly

greater IFN-γ than NTD CAR-T cells did. These results demonstrate that GMP grade anti-HLA-G CAR-T cells are highly potent *in vitro*.

[00435] The effect of increasing concentrations of soluble HLA-G on the potency of GMP grade anti-HLA-G CAR-T cells was assessed *in vitro*. It was reported that increased plasma levels of soluble HLA-G were found in patents with different tumors. Thus, the effect of increasing concentrations of soluble HLA-G on the potency of GMP grade anti-HLA-G CAR-T cells derived from the three donors was assessed by an *in vitro* cytotoxicity assay. Anti-HLA-G CAR-T cells were incubated on LCL cells expressing HLA-G (LCL-HLA-G) with the presence of increasing concentrations of soluble HLA-G. FIGS. 9A-9C show that up to 10 μg/ml soluble HLA-G had no effect on the cytotoxicity of anti-HLA-G CAR-T cells against HLA-G-expressing LCL cells *in vitro*.

Example 5-Validation of expression of HLA-G in tumor cells

[00436] A validation study using HLA-G IHC assay to specifically detect HLA class I histocompatibility antigen, alpha chain G, in human tissues was conducted to identify HLA-G expressing tumor cells in tissue sections from Formalin-Fixed, Paraffin-Embedded (FFPE) samples.

[00437] FFPE human tissue blocks include normal placenta, clear cell renal cell carcinoma (ccRCC), epithelial ovarian carcinoma (EOC), pancreatic cancer (PCA), colorectal cancer (CRC), esophageal cancer (ESC), breast cancer and (BRCA) tissue blocks. Anti-HLA-G (4H84) mouse monoclonal antibody was used in the HLA-G IHC assay. Interpretation of HLA-G expression in ccRCC, EOC, PCA, CRC, ESC, BRCA, normal tissues and controls was performed by board-certified licensed anatomic pathologists using conventional light microscopy.

[00438] Table 19 shows the HLA-G positivity rate per indication. High incidence of HLA-G expression was found in ccRCC and EOC samples. The IHC data support ccRCC and EOC as lead indications for the anti-HLA-G CAR T cell therapy.

Table 19. Anti-HLA-G CAR-T cell drug product stability Program Plan

Indication	Total # of samples stained	% of cases showing positive staining
ccRCC	40	85%
EOC	10	70%
ESC	10	40%
BRCA	10	40%
PCA	10	60%

CRC	10	50%

[00439] In a separate study, HLA-G isoforms in patient RCC biopsies were determined using a RT-PCT assay. The results showed that HLA-G1 and HLA-G5 were the main isoforms expressed in RCC tumors. Low level of HLA-G6 and no expression of HLA-G2 and HLA-G4 were detected in RCC tumors. The data indicate that humanized LFTT-1 CAR T cells that specifically binds to HLA-G1 and HLA-G5 would be effective for the treatment of RCC.

CLAIMS

What is claimed is:

1. A chimeric antigen receptor (CAR) comprising:

(A) an extracellular domain comprising an antigen binding domain that specifically binds to human leukocyte antigen G (HLA-G), wherein the antigen binding domain comprises:

(a) (i) a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 25; and (ii) a light chain variable region (VL) comprising the sequence of SEQ ID NO: 35;

(b) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 50; or

(c) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 60;

(B) a transmembrane domain; and

(C) an intracellular signaling domain.

2. A CAR comprising:

(A) an extracellular domain comprising an antigen binding domain that specifically binds to HLA-G, wherein the antigen binding domain comprises:

(a) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 25; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 35;

(b) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 50; or

(c) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 60;

(B) a transmembrane domain; and

(C) an intracellular signaling domain.

3. The CAR of claim 1 or 2, wherein the antigen binding domain is a single chain variable fragment (scFv).

4. The CAR of claim 3, wherein the antigen binding domain comprises a sequence set forth in one of SEQ ID NOs: 63, 65, and 67.

5. The CAR of claim 1 or 2, wherein the transmembrane domain is a CD28 transmembrane domain; and wherein the intracellular signaling domain comprises a 4-1BB costimulatory signaling region and a CD3 zeta endodomain.

6. The CAR of claim 5, wherein:

the CD28 transmembrane domain comprises the sequence of SEQ ID NO: 72,

the 4-1BB costimulatory signaling region comprises the sequence of SEQ ID NO: 73, and

the CD3 zeta endodomain comprises the sequence of SEQ ID NO: 74.

7. The CAR of claim 1 or 2, wherein the intracellular signaling domain further comprises a CD28 costimulatory domain.

8. The CAR of any of claims 1-7, further comprising a signal peptide.

9. The CAR of claim 8, wherein the signal peptide comprises the sequence of SEQ ID NO: 69.

10. The CAR of any of claims 1-9, further comprising a hinge domain connecting the antigen binding domain to the transmembrane domain.

11. The CAR of claim 10, wherein the hinge domain comprises the sequence of SEQ ID NO: 71.

12. The CAR of any of claims 1-11, further comprising a cleavable linker.

13. The CAR of claim 12, wherein the cleavable linker comprises the sequence of SEQ ID NO: 75.

14. The CAR of any of claims 1-13, further comprising a truncated human CD19 domain.

15. The CAR of claim 14, wherein the truncated human CD19 comprises the sequence of SEQ ID NO: 76.

16. The CAR of claim 1 or 2, wherein the CAR comprises the sequence of SEQ ID NO: 68.

17. A nucleic acid molecule encoding the CAR of claim 1 or 2.

18. An expression vector comprising the nucleic acid molecule of claim 17.

19. A cell comprising the nucleic acid molecule of claim 17.

20. A cell comprising the expression vector of claim 18.

21. A cell comprising the CAR of claim 1 or 2.

22. The cell of claim 21, wherein the cell is a T cell, a B cell, a NK cell, a NKT cell, a monocyte cell or a dendritic cell.

23. A pharmaceutical composition comprising the cell of any of claims 19-22, and a pharmaceutically acceptable carrier.

24. The pharmaceutical composition of claim 23, wherein the cell comprises no more than five copies of the expression vector in each transduced cell.

25. The pharmaceutical composition of claim 23, further comprising no less than 70 percent viable cells.

26. The pharmaceutical composition of claim 23, further comprising no more than

5EU/kg endotoxin.

27. The pharmaceutical composition of claim 23, further comprising less than 50 copies/μg replication-competent lentivirus.

28. The cell of any of claims 19-22 for use in the treatment of cancer.

29. The pharmaceutical composition of any of claims 23-27 for use in the treatment of cancer.

30. A method for treating a cancer in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition of claim 29.

31. The method of claim 30, wherein the cancer is a solid tumor.

32. The method of claim 30, wherein the cancer is a hematological cancer.

33. The method of claim 30, wherein the cancer is clear cell renal cell carcinoma, epithelial ovarian carcinoma, melanoma, kidney cancer, bladder cancer, breast cancer, ovarian cancer, uterine cancer, endometrial cancer, cervical cancer, renal cell cancer, colorectal cancer, gastric cancer, esophageal cancer, lung cancer, hepatocellular cancer, cholangiocarcinoma, neuroblastoma, cancer of the tongue, mouth and pharynx, bronchogenic cancer, cancer of the larynx, osteosarcoma, prostate cancer, testicular cancer, gastrointestinal stromal tumor, pancreatic cancer, kidney cancer, colon cancer, glioma, glioblastoma multiforme, medulloblastoma, thyroid cancer, adrenal carcinoma, acute myeloid leukemia, chronic lymphocytic leukemia, non-small cell lung cancer, multiple myeloma, Hodgkin's lymphoma, non-Hodgkin lymphoma, B-cell lymphoma, monocytic lymphoma, marginal zone lymphoma, Burkitt's lymphoma, T cell lymphoma, B cell lymphoma, plasmacytoma, prohemocytic leukemia, acute non-lymphoblastic leukemia, acute lymphoblastic leukemia, erythroleukemia, myeloid leukemia or lymphoid leukemia.

34. The method of claim 30, wherein the cell is autologous.

35. The method of claim 30, wherein the cell is allogeneic.

36. The method of claim 30, wherein the pharmaceutical composition is administered to the subject by intravenous, intratumoral, intraperitoneal, intramuscular, intraportal, intrahepatic, subcutaneous or intradermal administration.

37. The method of claim 30, wherein the subject is human.

38. An anti-HLA-G antibody or an antigen-binding fragment thereof, wherein the antibody or antigen-binding fragment comprises:

(a) (i) a VH comprising the sequence of SEQ ID NO: 25; and (ii) a VL comprising the sequence of SEQ ID NO: 35;

(b) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 50; or

(c) (i) a VH comprising the sequence of SEQ ID NO: 9; and (ii) a VL comprising the sequence of SEQ ID NO: 60.

39. An anti-HLA-G antibody or an antigen-binding fragment thereof, wherein the antibody or antigen-binding fragment comprises:

(a) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 25; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 35;

(b) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 50; or

(c) (i) a VH comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9; and (ii) a VL comprising a sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 60.

40. The antibody or antigen-binding fragment of claim 38 or 39, wherein the antibody or

antigen-binding fragment is chimeric.

41. The antibody or antigen-binding fragment of any of claims 38-40, wherein the antibody or antigen-binding fragment is conjugated to a toxic agent.

42. The antigen-binding fragment of any of claims 38-41, wherein the antigen-binding fragment is a scFv, a Fv, a Fab, a Fab', or a F(ab')2.

43. The antibody or antigen-binding fragment of any of claims 38-42, wherein the antibody is monoclonal.